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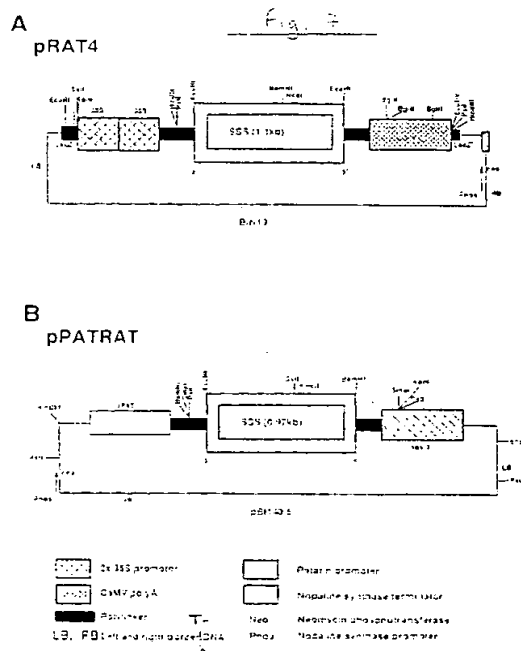
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(54) Improvements in or relating to soluble starch synthase

(57) Disclosed is altered starch extracted from a transformed potato plant or the progeny thereof, which starch, as extracted, has a viscosity onset temperature as determined by differential scanning calorimetry which is reduced by at least 5°C compared to starch extracted from equivalent, non-transformed plants, together with a nucleic acid sequence comprising at least 200bp and exhibiting at least 80% sequence identity with the corresponding region of the DNA sequence shown in Figure 6, operably linked in the sense or anti-sense orientation to a promoter operable in a plant, and host cells and plants comprising the sequence.



## Description

### Field of the Invention

This invention relates, *inter alia*, to a soluble enzyme, obtainable from potato tubers, having starch synthase activity, to nucleic acid sequences encoding the same, to constructs and transgenic plants comprising the nucleic acid sequences, to a method of altering the starch composition of a plant, and to altered starch obtainable from a transgenic plant.

### Background of the Invention

In the storage organs of most species of plants multiple forms of both granule-bound and soluble starch synthases have been found (for review, see Smith & Martin 1993, In: Biosynthesis and manipulation of plant products (D. Grierson, Ed.) Blackie Academic and Professional (Glasgow), pp1-54). In most cases it is not known whether these forms are distinct gene products and, for the most part, what their detailed functions are. The exception to this is in the case of a widely-distributed and highly conserved class of granule-bound starch synthases of approximately 60 kDa, which are collectively referred to as granule-bound starch synthase I (GBSS I; Martin & Smith, 1995 Plant Cell 7, 971-985). Experiments with the waxy and amf mutants of cereals and potatoes respectively (Macdonald & Preiss, 1985 Plant Physiol. 78, 849-852 1985; Hovenkamp-Hermelink *et al.*, 1987 Theor. Appl. Genet. 7, 217-221) and antisense potato plants (Visser *et al.*, 1991 Mol. Gen. Genet. 22, 289-296, Kuipers *et al.*, 1994 Plant Cell 6, 43-52) have shown that when the level of GBSS I protein is reduced, the ratio of amylose to amylopectin in the starch is also reduced. Where GBSS I is absent, the starch contains only amylopectin. This suggests that GBSS I is responsible for amylose synthesis.

However, the detailed functions of other isoforms of starch synthase are as yet unknown. In general, in conjunction with starch branching enzyme, they must be responsible for amylopectin synthesis but it is unknown whether different isoforms make different contributions to its structure. The first step in trying to understand the functions of these starch synthases is to characterise all of the isoforms in one organ. A few isoforms of starch synthase, other than GBSS I, have been identified at a detailed biochemical and molecular level in pea (Smith, 1990 Planta 182, 599-604; Denyer & Smith 1992 Planta 186, 609-617, Dry *et al.* 1992 Planta 186, 609-617) and rice (Baba *et al.*, 1993 Plant Physiol. 103, 565-573), and at a detailed biochemical level in maize (Mu *et al.*, 1994 Plant J. 6, 151-159) and wheat (Denyer *et al.*, 1995 Planta 196, 256-265). However only in the case of pea and maize has the quantitative importance of the isoforms been estimated. A complete picture of the role and importance of all the isoforms of starch synthase is not available for any other storage organ.

Carbohydrate metabolism and starch synthesis has been extensively studied in potato tuber (Hajirezaei *et al.*, 1993 Planta 192, 16-30; Geigenberger & Stitt 1993 Planta 189, 329-339; Geigenberger *et al.*, 1994 Planta 193, 486-493; Sonnewald *et al.*, 1994 Plant Cell Environ. 17, 649-658) and this organ has great potential as a source of commercially important starches created through genetic manipulation (Shewmaker & Stalker 1992 Plant Physiol. 100, 1083-1086; Visser & Jacobsen 1993 Trends Biotech. 11, 63-68; Muller-Rober & Koßmann, 1994 Plant Cell Environ. 17, 601-613). One of the major gaps in understanding starch synthesis in this organ and hence in the ability to manipulate its starch in useful ways, is the nature of its starch synthases.

In potato, until recently, only two starch synthases have been characterised in any great detail; GBSS I and GBSS II. GBSS I is exclusively granule-bound, it has a molecular weight of 59 kDa. The gene has been cloned and its predicted amino acid sequence is very similar to that of the waxy gene product in cereals (Vos-Scheperkeuter *et al.*, 1986 Plant Physiol. 82, 411-416; van der Leij *et al.*, 1991 Mol. Gen. Genet. 228, 240-248). GBSS II has an apparent molecular weight, as judged by SDS-PAGE, of 92 kDa and it is both bound into the starch granule and present as a soluble form. Its predicted amino acid sequence (having an expected molecular weight of 80kDa) is similar to GBSS II in pea embryos, an isoform which accounts for 60-70 % of the soluble starch synthase activity of the pea embryo (Denyer & Smith 1992 cited above). However, GBSS II accounts for only approximately 10-15% of the total soluble starch synthase activity in potato tubers (Edwards *et al.*, 1995 Plant J. 8, 283-294).

There have been several reported characterisations of the starch synthases found in the soluble fraction of potato tubers (Frydman & Cardini 1966 Arch. Biochem. Biophys. 116, 9-18; Catz *et al.*, 1989 An. Asoc. Quim. Argent. 77, 47-51) and a few attempts have been made to purify the major soluble starch synthases (Hawker *et al.*, 1972. Phytochem. 11, 1278-1293; Baba *et al.*, 1990 Phytochem. 29, 719-723; Ponstein 1990. Starch synthesis in potato tubers. Ph. D. Thesis, State University Groningen, The Netherlands). These reports disagree on both the number of soluble starch synthases and their molecular weights. The quantitative contribution of the putative forms is not known, and where multiple forms are postulated, it is not known whether they are independent gene products.

After the priority date of the present application, two publications have been made which provide information about a further starch synthase found in potato. One of these publications is by the present inventors (Marshall *et al.*, 1996 The Plant Cell 8, 1121-1135). The other publication is PCT patent application WO 96/15248 (published 23rd May 1996),

in the name of Institut Für Genbiologische Forschung Berlin GMBH. The PCT application includes the European Patent Office in the list of designated territories.

WO 96/15248 discloses the nucleotide sequence of a full length cDNA clone ("SSSA") said to encode an isoform of soluble starch synthase enzyme from potato, together with the predicted amino acid sequence of the enzyme. The application further discloses the use of a 1.2kb portion of the cDNA clone, operably linked in the antisense orientation to the CaMV 35S promoter, to transform potato plants. In addition WO 96/15248 discloses the sequence of a cDNA clone ("SSSB") said to encode a second isoform of the potato soluble starch synthase. Similarly, a portion (1.8kb) of this sequence was introduced into potato plants in the antisense orientation.

In fact, the present inventors have found that the nucleotide sequence disclosed in WO 96/15248 contains an error, causing a frame shift, such that most of the predicted amino acid sequence is incorrect.

It was found that the transformed plants disclosed in WO 96/15248 had reduced enzyme activity. Starch obtained from the tubers of the transformed plants was found to have altered properties compared to starch obtained from control wild type plants. It was stated that the starch from the transformed plants exhibited a lower viscosity onset temperature than starch from control plants. (By way of explanation, when aqueous suspensions of starch granules are heated, the granules swell and absorb water, in a process known as gelatinisation. A number of techniques are available for the analysis of gelatinisation, a particularly convenient method being differential scanning calorimetry or the viscoamylograph, in which the viscosity of a stirred starch suspension is monitored under a defined temperature/time regime. Such analysis typically shows a particular temperature, the "viscosity onset temperature", at which the process of gelatinisation begins and which causes a marked increase in viscosity of the starch suspension).

In a few instances, the transformed plants disclosed in WO 96/15248 gave rise to starch in which the "Verkleisterungstemperatur" (equivalent to the viscosity onset temperature, V) was 2 to 3°C lower compared to starch from equivalent, but untransformed, plants. However, it is apparent that the results of subsequent experiments (described in example 13 in the document) gave a value of V for starch from control plants which was lower than that found for starch from transformed plants in previous experiments. Accordingly, the person skilled in the art is not able to deduce that starch from the transformed plants described in WO 96/15248 displayed a viscosity onset temperature which was consistently significantly lower than that of control plants.

### Summary of the Invention

In a first aspect the invention provides a polypeptide obtainable from a soluble extract of potato tubers and having starch synthase activity, in substantially isolated form.

Typically the polypeptide will have an apparent molecular weight, as judged by SDS-PAGE, in the range 100-140 kDa, or will be a functional equivalent of such a polypeptide. More particularly, the polypeptide may have an apparent molecular weight of 140, 120 or 110 kDa. Particular functional equivalents envisaged are breakdown products of the polypeptide, which seem to occur naturally. Another particular functional equivalent is the polypeptide obtainable from developing tubers of the Desiree cultivar, which polypeptide has an apparent molecular weight, as judged by SDS-PAGE, of 100 kDa. Typically the polypeptide will comprise the amino acid sequence shown in Figure 6.

In another aspect the invention provides a nucleic acid sequence directing the expression of at least a portion of one of the polypeptides defined above. Preferably the sequence comprises at least 200-300bp, more preferably at least 300-600bp, and most preferably in excess of 600bp. Typically the nucleic acid sequence will comprise the nucleotide sequence shown in Figure 6, although those skilled in the art will appreciate that, due to the degeneracy of the genetic code, a nucleotide sequence substantially different to that shown in Figure 6 may encode a polypeptide having substantially the same amino acid sequence as that shown in Figure 6. Such nucleic acid sequences are to be considered as functional equivalents and thus fall within the scope of the present invention. Other functional equivalents are those nucleic acid sequences which are not substantially different and which may hybridise, under standard laboratory hybridisation conditions, to either strand of the nucleotide sequence shown in Figure 6.

Comparison with known starch synthase sequences, with the benefit of the disclosure herein, will enable those skilled in the art to identify regions of the sequence shown in Figure 6 which are not evolutionarily conserved, and so more amenable to alteration (e.g. addition, deletion or substitutions), whilst retaining functional equivalence.

Desirably such functional equivalents will possess at least 80% sequence identity, preferably at least 85% sequence identity, and more preferably at least 90% sequence identity with the nucleotide sequence shown in Figure 6. Desirably the nucleotide sequence of the invention, or a functional equivalent sequence will, when introduced into a suitable plant in a suitable manner (known to those skilled in the art), alter the synthesis of starch in the plant.

For the purposes of the present specification, the sequences encoding polypeptides with starch synthase activity, or portions of such sequences, disclosed in WO 96/15248 are not considered as functional equivalents of the sequence shown in Figure 6.

In a particular embodiment, the invention provides a nucleic acid sequence comprising at least 200 bp and exhibiting at least 80% sequence identity with the corresponding region of the DNA sequence shown in Figure 6, operably

linked in the sense or antisense orientation to a promoter operable in a plant.

Those skilled in the art will readily be able to conduct a sequence alignment between the other sequence and that detailed in Figure 6. The % identity of the two sequences is to be compared in those regions which are aligned by readily available computer programs (e.g. MegAlign), which align corresponding regions of sequences. Advantageously the % identity between the two sequences will be at least 85%, preferably at least 90%, and the corresponding region of the sequence shown in Figure 6 may comprise a 5' and/or a 3' untranslated region ("UTR") and/or a translated region.

Thus, in another aspect the invention provides a nucleic acid construct (typically DNA) comprising the nucleic acid sequence of the invention in operable linkage to a promoter active in a plant. The nucleic acid sequence may be operably linked to the promoter in either the sense or the anti-sense orientation. Anti-sense methods are well known in altering one or more characteristics of a plant into which the anti-sense sequence is inserted (see for example EP-A-0 458 367, EP-B-0 240 208 and US 5, 107, 065). Similarly, "sense suppression" is a method which is becoming increasingly widely adopted and documented (for a review, see Matzke & Matzke 1995 Plant Physiology 107, 679-685). Either approach could be used with the nucleic acid sequence of the invention, so as to alter one or more characteristics of a plant into which the sequence was introduced. Those skilled in the art will be aware that anti-sense inhibition or sense suppression may be achieved by the use of 5' or 3' non-translated portions of the relevant gene, or use of coding portions of the gene, or any combination thereof.

It is believed that antisense methods are mainly operable by the production of antisense mRNA which hybridises to the sense mRNA, preventing its translation into functional polypeptide, possibly by causing the hybrid RNA to be degraded (e.g. Sheehy *et al.*, 1988 PNAS 85, 8805-8809; Van der Krol *et al.*, Mol. Gen. Genet. 220, 204-212). Sense suppression also requires homology between the introduced sequence and the target gene, but the exact mechanism is unclear. It is apparent however that, in relation to both antisense and sense suppression, neither a full length nucleotide sequence, nor a "native" sequence is essential. Preferably the nucleic acid sequence used in the method will comprise at least 200-300bp, more preferably at least 300-600bp, of the full length sequence, but by simple trial and error other fragments (smaller or larger) may be found which are functional in altering the characteristics of the plant. It is also known that untranslated portions of sequence can suffice to inhibit expression of the homologous gene - coding portions may be present within the introduced sequence, but they do not appear to be essential under all circumstances.

The invention further provides a host cell into which has been introduced a nucleic acid sequence in accordance with the invention defined above. Typically the host cell will be a plant cell, and conveniently the sequence is introduced in a nucleic acid construct and subsequently integrated into the host cell genome.

In a further aspect the invention provides a plant or part thereof (e.g. plant cell), into which has been introduced a sequence in accordance with the invention, or the progeny of such a plant or part thereof. Desirably the plant or part thereof into which the sequence is introduced, will comprise a natural gene which shares sequence homology with the introduced sequence. In preferred embodiments the introduced sequence will exhibit at least 70% homology with a starch synthase gene naturally present in the plant or part thereof, although the level of homology may be increased with advantage, such that the expression of the gene product of the naturally present gene in the plant is substantially inhibited. Conveniently the sequence of the invention will be introduced as part of a nucleic acid construct, as described above. Typically the plant will be one of commercial significance, such as one of the following: potato, tomato, rice, wheat, pea, cassava, sweet potato, barley, oat and maize.

Those skilled in the art will appreciate that introduction of the nucleic acid sequence of the invention into a plant might alter the starch composition thereof. In another aspect therefore the invention provides altered starch extracted from a plant into which has been introduced the nucleic acid sequence of the invention, or altered starch extracted from the progeny of such a plant. The invention also provides a method of altering one or more characteristics of a plant, comprising introducing into the plant a nucleic acid sequence in accordance with the invention.

In particular the invention provides altered starch extracted from a transformed potato plant or the progeny thereof, which starch, as extracted, has a viscosity onset temperature as determined by differential scanning calorimetry which is reduced by at least 5°C compared to starch extracted from equivalent, non-transformed plants. Preferably the viscosity onset temperature is reduced by at least 7°C. In another embodiment the invention provides altered starch extracted from a transformed potato plant or the progeny thereof, which starch, as extracted, has a viscosity onset temperature as determined by differential scanning calorimetry, of less than 60°C, preferably less than 55°C.

The starch defined above will typically also exhibit (as extracted) a reduced endotherm peak temperature, as determined by differential scanning calorimetry, compared to starch extracted from equivalent, non-transformed plants. Desirably the endotherm peak temperature will be reduced by at least 5°C and/or will be less than 59°C. The inventors have found that such properties as those defined above may be embodied in potato starch having a substantially normal amylose content (i.e. around 25 - 30% amylose).

Starch can be modified in various ways (e.g. chemical cross-linking, derivatisation, partial hydrolysis) after it has been extracted from a plant source, which modifications can affect the physical properties, especially the pasting properties, of the starch. Hence, use of the term "as extracted" is intended to signify that the starch is analysed without

undergoing such modifications as can alter the pasting properties thereof.

"Equivalent, non-transformed" plants are those plants which have substantially identical genotypes to the plants of the invention, with the exception of the introduced nucleic acid sequence present in the transformed plants of the invention.

The invention will now be further described by way of illustrative example and with reference to the accompanying drawings, in which;

Figure 1 shows the elution profile of starch synthase from developing Desiree potato tubers on a first Mono Q™ anion-exchange column. Partially purified starch synthase, after DEAE-Sepharose and Blue Sepharose chromatography, was applied to a 1ml Mono Q™ column at pH 7.5. The enzyme was eluted with a 25ml gradient of 0-450 mM KCl at 0.5 ml.min<sup>-1</sup>. Samples (20 µl) of each 1 ml fraction were assayed for starch synthase activity (●), and absorbance at 280 nm (○);

Figure 2 shows the activity and protein in fractions of purified starch synthase from a second Mono Q™ column of peak I and peak II. Top panels show SDS-PAGE of fractions containing starch synthase activity. Each track contains 10 µl of fraction. Bottom panels show starch synthase activity in 20 µl samples from each 0.5 ml fraction;

Figure 3 shows the cross-reaction of antiserum to SSS to the purified starch synthases from mature Estima tubers and to extracts, soluble and granule-bound, from mature Estima and developing Desiree tubers. Samples (10 µl of purified soluble starch synthase, 20 µl of partially purified soluble starch synthase, 20 µl soluble extract and 20 µl of supernatant from granule-bound proteins) were subjected to SDS-PAGE and blotted, and then the blots were probed with antiserum to SSS, 1/2500 dilution. (1) purified preparation of starch synthase proteins from mature Estima. (2) Partially purified soluble starch synthase from mature Estima tubers. (3) Starch-granule-bound proteins from mature Estima tubers. (4) Soluble extract from developing Desiree tubers. (5) Starch-granule-bound proteins from developing Desiree tubers. Sizes of proteins were estimated from molecular weight standards on the same gels, and are indicated in kDa;

Figure 4 shows the immunoprecipitation of starch synthase activity in soluble extract from developing Desiree tubers with antiserum to SSS. Soluble extract was incubated with increasing volumes of pre-immune serum (○) and antiserum (●), as described in Materials and Methods (below). After centrifugation the supernatant was assayed for starch synthase activity. Starch synthase activity is expressed as a percentage of activity of incubations containing 20 g.L<sup>-1</sup> BSA in PBS. Values are from two separate experiments with the line joining the means;

Figure 5 shows native polyacrylamide gel electrophoresis of soluble extract from developing Desiree tubers stained for starch synthase activity. Soluble extract was incubated (as described in Materials and Methods) in the presence of (1) 20 g.L<sup>-1</sup> BSA in PBS; (2) pre-immune serum, 1/1000 dilution; (3) antiserum to SSS, 1/1000 dilution; and (4) antiserum to the GBSS II from pea embryo. After centrifugation, the supernatant was mixed 5:1 with 2 g.L<sup>-1</sup> bromophenol blue in 500 ml.L<sup>-1</sup> glycerol and 40 µl was loaded onto the gel. The bands of starch synthase activity are indicated by arrows;

Figure 6 shows the DNA sequence of a cDNA clone for potato soluble starch synthase. The amino acid sequence of the encoded polypeptide is shown below in the single letter code. The ADP-glucose binding domain is boxed and the sequences identified by protein sequencing are underlined; and

Figure 7 shows a schematic representation of A) plasmid pRAT4 and B) plasmid pPATRAT.

## EXAMPLES

### Example 1

In this example are presented data on the identification and purification to homogeneity of the major isoform of soluble starch synthase from potato tuber.

## MATERIALS AND METHODS

### Plant material.

Potato tubers (*Solanum tuberosum* L.) of cultivars Desiree (developing) or Estima (mature) were used. Desiree

tubers were grown in pots of soil based compost (25 cm diameter) in a greenhouse with minimum temperature of 12°C and supplementary lighting in winter, and were freshly harvested prior to experiments from actively-growing plants. Estima tubers were bought locally.

## 5 Purification of soluble starch synthase.

(A) Small scale. The extraction and subsequent purification were carried out at 4°C. Approximately 500g of Desiree potato tubers were chopped into small pieces and homogenised in an electric blender with 25g polyvinylpyrrolidone (PVPP) and 500 ml of ice-cold medium A containing 100 mM Tris-HCl (pH 7.5), 10 mM ethylenediamine-tetraacetic acid (EDTA), 5 mM dithiothreitol (DTT), 1 g.L<sup>-1</sup> sodium metabisulphite, 0.5 mg.L<sup>-1</sup> leupeptin, 0.7 mg.L<sup>-1</sup> pepstatin A and 50 ml.L<sup>-1</sup> glycerol. The homogenate was passed through two layers of muslin and the filtrate was centrifuged at 10,000g for 10 min. The supernatant was brought to 40% saturation with powdered (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. The precipitate was collected by centrifugation (15,000g for 15 min), re-dissolved in a minimal volume of medium A and dialysed twice, each time against 1L of medium A for 1 h.

15 The dialysed extract was applied, at a flow rate of 4 ml.min<sup>-1</sup>, to a column (5 cm internal diameter "i.d.", 10 cm long) of diethylaminoethyl (DEAE)-Sephacel™ (Pharmacia, Uppsala, Sweden), equilibrated with medium A. The column was washed with 500 ml of medium A followed by a 250-ml gradient of 0-1 M KCl in the same medium. Fractions of 10 ml were collected and assayed for starch synthase activity. The eight to ten fractions containing the highest activity were pooled and dialysed twice, each time against 1 L of medium B containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, 0.5 mg.L<sup>-1</sup> leupeptin, 0.7 mg.L<sup>-1</sup> pepstatin A and 50 ml.L<sup>-1</sup> glycerol for 1 h.

20 The dialysed extract was applied, at a flow rate of 1 ml.min<sup>-1</sup>, to a column (1.6 cm i.d., 16 cm long), of Blue Sepharose, equilibrated with medium B. The column was washed with 100 ml medium B followed by a 100ml gradient of 0-1 M KCl in the same medium. Fractions of 0.5 ml were collected and assayed for starch synthase activity. The ten fractions with the highest activity were pooled and dialysed twice, each time against 1 L of medium B for 1 h.

25 The dialysed extract was applied, at a flow rate of 0.5 ml.min<sup>-1</sup>, to a first 1-ml Mono Q™ column (Pharmacia), equilibrated with medium B. The column was washed with 25 ml of medium B, followed by a 25-ml gradient of 0-450 mM KCl in the same medium. Fractions of 0.5 ml were collected and assayed for starch synthase activity. The fractions from each of two peaks of starch synthase activity were pooled and purified separately as follows:

30 To the eluate of the first Mono Q™ column, an equal volume of 1 M sodium citrate in medium B was added. This was then applied, at a flow rate of 0.5 ml.min<sup>-1</sup>, to a column (1.0 cm i.d., 4 cm long) of cyclohexa-amylose (CHA)-Sephacel™ (prepared according to Vretblad, 1974 FEBS Lett. 47, 86-89), equilibrated with 0.5 M sodium citrate in medium B. The column was washed with 20 ml medium B containing 0.5 M sodium citrate and the protein was eluted from the column with 30 ml of medium B containing no citrate. Fractions of 1 ml were collected and assayed for starch synthase activity. The fractions with starch synthase activity were pooled and dialysed overnight against 5 L of medium C containing 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM DTT, 0.5 mg.L<sup>-1</sup> Leupeptin, 0.7 mg.L<sup>-1</sup> pepstatin A and 50 ml.L<sup>-1</sup> glycerol.

35 The dialysed extract was applied, at a flow rate of 0.5 ml.min<sup>-1</sup>, to a second 1ml Mono Q™ column equilibrated with medium C. The column was washed with 25 ml of medium C followed by a 25ml gradient of 0-450 mM KCl in the same medium. Fractions of 0.5 ml were collected and assayed for starch synthase activity.

## 40 (B) Large scale.

The procedures were as described above, with the following modifications. Five kg of Estima potato tubers were homogenised in 5 L medium A containing 250g PVPP, filtered through two layers of muslin and centrifuged at 10,000g for 10 min. Polyethylene glycol (PEG) 6000 at a concentration of 500 g.L<sup>-1</sup> in medium A was slowly added to the supernatant until the concentration of PEG was 100 g.L<sup>-1</sup>. The precipitate was collected by centrifugation (15,000g for 20 min) and re-dissolved in a minimal volume of medium A.

45 The extract was mixed gently for 1 h with 900ml slurry of DEAE-Sephacel™ which had been equilibrated with medium A, then filtered and the filtrate discarded. The DEAE-Sephacel™ was washed with 2 L medium A then incubated for 1 hr in 500 ml medium A containing 400 mM KCl, filtered and washed with a further 500 ml medium A containing 400 mM KCl. The filtrates were combined and brought to 50% saturation with powdered (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. The precipitated proteins were collected by centrifugation (15,000g for 15 min), re-dissolved in a minimal volume of medium B and dialysed overnight against 5 L of medium B.

55 The dialysed sample was applied, at a flow rate of 2 ml.min<sup>-1</sup>, to a Blue Sepharose column (5 cm i.d., 15 cm long) which had been equilibrated with medium B. The column was washed with 300 ml medium B followed by a 600ml gradient of 0-1 M KCl in the same medium, at a flow rate of 5 ml.min<sup>-1</sup>. Fractions of 15 ml were collected and assayed for starch synthase activity. The ten fractions with the highest starch synthase activity were pooled and dialysed overnight against 5 L medium B.

The dialysed eluate was applied to a first 1ml Mono Q™ column, equilibrated with medium B, as described above, except that all the fractions containing starch synthase activity were pooled together.

The Mono Q™ eluate was applied to a CHA-Sepharose column as described above, except that the column was 1.0 cm i.d., 20 cm long. The column was washed with 50 ml medium B containing 0.5 M sodium citrate and eluted with 80 ml medium B without citrate. The fractions with starch synthase activity were pooled and dialysed overnight against 5 L of medium C.

The dialysed extract was applied to a second 1ml Mono Q™ column equilibrated with medium C, as described above. Fractions containing starch synthase activity were stored at -20°C.

#### Preparation of antibody.

The fractions containing starch synthase activity from 5 large-scale purifications were run on preparative sodium dodecyl sulphate (SDS)-polyacrylamide gels (as described below). The gel slices containing starch synthase proteins were electroeluted and the proteins dialysed against water, then freeze-dried. Protein (50 µg) was re-dissolved in 250 µl of phosphate-buffered saline (PBS), mixed with 250 µl Freund's complete adjuvant, and injected intramuscularly into a rat. Subsequent injections were of 75 µg protein dissolved in 250 µl PBS mixed with 250 µl Freund's incomplete adjuvant and were repeated at 14-day intervals. Serum was collected from 14 days after the third injection.

#### Assay of soluble starch synthase activity.

Soluble starch synthase activity was measured using the resin method as described in Jenner *et al.* (1994).

#### Preparation of crude soluble potato tuber extract.

Samples (0.5-2.0 g fresh weight) from either developing Desiree or mature Estima potato tuber were homogenised in 4 volumes of 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, 1 g.L<sup>-1</sup> sodium metabisulphite, 0.5 mg.L<sup>-1</sup> leupeptin, 0.7 mg.L<sup>-1</sup> pepstatin A and 50 ml.L<sup>-1</sup> glycerol, then centrifuged at 10,000g for 10 min. The supernatant is referred to as "soluble extract".

#### Partial purification of soluble starch synthase activity.

Crude soluble potato extract from mature Estima tubers (5-10 g fresh weight) was dialysed twice, each time against 1 L of buffer B for 1 hr. The dialysed extract was applied to a 1ml Mono Q™ column, equilibrated with medium B, as described above and the peak fraction of starch synthase activity (referred to as "partially purified soluble starch synthase") was stored at -20°C.

#### SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting.

Protein samples were dialysed against distilled water then mixed 1:1 with double-strength sample buffer (Laemmli, U.K. (1970). Nature 227, 680-685) and boiled for 2 min immediately prior to application to gels. For granule-bound proteins, starch granules were washed twice in 20 g.L<sup>-1</sup> SDS at room temperature, boiled for 3 min at 100 mg.ml<sup>-1</sup> in sample buffer (Laemmli, U.K. (1970). Nature 227, 680-685) and then centrifuged at 10,000g for 10 min. The supernatant was applied to the gel.

Gels (10.2 cm long, 7.3 cm wide, 0.75 mm thick) were 75 g.L<sup>-1</sup> acrylamide (37.5:1 w/w acrylamide:bis-acrylamide) and 1 g.L<sup>-1</sup> SDS and were run according to Laemmli (1970). Immunoblots were prepared and developed according to Bhattacharyya *et al.*, (1990) Cell 60, 115-122. The nitrocellulose filters were either incubated with crude rat serum followed by alkaline phosphatase-conjugated goat anti-rat antiserum (Sigma, Poole, Dorset, UK) or the immunoglobulin fraction of rabbit serum to GBSS II from pea embryo (Smith (1990) Planta 182, 599-604), followed by alkaline phosphatase-conjugated goat anti-rabbit antiserum (Sigma).

#### Native polyacrylamide gel electrophoresis.

Gels (dimension as above, except 1mm thick) of 90 g.L<sup>-1</sup> acrylamide (37.5:1 w/w acrylamide:bis-acrylamide) were cast in 400 mM Tris-HCl (pH 8.6), 100 ml.L<sup>-1</sup> glycerol, 8 g.L<sup>-1</sup> glycogen and polymerised with 0.4 g.L<sup>-1</sup> ammonium persulphate and 0.2 ml.L<sup>-1</sup> N,N,N',N'-tetramethylethylenediamine (TEMED) and were overlaid with a stacking gel of 53 g.L<sup>-1</sup> acrylamide (37.5:1 w/w acrylamide:bis-acrylamide) cast in 155 mM Tris-HCl (pH 6.8), 98 ml.L<sup>-1</sup> glycerol, polymerised with 0.5 g.L<sup>-1</sup> ammonium persulphate and 0.2 ml.L<sup>-1</sup> TEMED. Soluble extracts were mixed 5:1 with 2 g.L<sup>-1</sup> bromophenol blue in 500 ml.L<sup>-1</sup> glycerol immediately prior to loading. Gels were run at 4°C, at 175 mV in 190 mM

glycine, 25 mM Tris.

The gel was assayed for starch synthase activity as follows. The gel was washed twice, each time for 10 min in 20 ml 100 mM Bicine, 0.5 M sodium citrate (pH 8.5), 0.5 M EDTA and 100 ml.L<sup>-1</sup> glycerol at 4°C. The gel was incubated at room temperature for 20 hrs by gently shaking in wash medium containing 12 mM ADPG and 2 mM DTT. The buffer was removed and 1 ml of Lugol's iodine solution (3.3 g.L<sup>-1</sup> I<sub>2</sub> and 6.7 g.L<sup>-1</sup> KI, acidified with a few drops of 2M HCl) was added. After colour development, the gel was washed and stored in 70 ml.L<sup>-1</sup> acetic acid.

#### Immunoprecipitation.

Soluble extracts (100µl) were incubated with 0-20µl rat serum or 20µl of the immunoglobulin fraction of rabbit serum to GBSS II from pea embryo (Smith (1990) Planta 182, 599-604) for 1.5 h at room temperature on a rotating table. To the extract containing rat serum, 20µl polyclonal antiserum to rat IgG at 2.5 g.L<sup>-1</sup> specific antibody (Sigma) was added and incubated for a further 0.5 h. To both extracts, 50 µl Protein A-Sepharose at 60 g.L<sup>-1</sup> in 50 mM Tris-HCl (pH 7.5) was added and then incubated for 0.5 h, followed by centrifugation at 10,000g for 10 min. The supernatants were assayed for starch synthase activity. Controls contained bovine serum albumin at 20 g.L<sup>-1</sup> in PBS in place of serum.

#### Isolation of starch granules.

Purified starch was prepared from potato tubers as described by Edwards *et al.* (1995).

#### Measurement of protein.

Protein was assayed using the BioRad Protein Assay Dye Reagent (BioRad Munchen, Germany) with a standard curve of bovine serum albumin.

## RESULTS

#### Purification of soluble starch synthases.

The soluble starch synthase activity from developing tubers of Desirée and mature tubers of Estima eluted from both DEAE-Sepharose and Blue Sepharose columns as a single peak of activity. However, subsequent chromatography on a Mono Q™ column at pH 7.5 separated two major peaks of starch synthase activity, designated peak I and peak II according to their elution order from the column (Figure 1). These two peaks of starch synthase activity were then purified separately by cyclohexa-amylose and Mono Q™ chromatography. A typical purification from developing Desirée tubers is shown in Table 1. The specific activity of peak I was 5.1 µmol.(mg protein)<sup>-1</sup>.min<sup>-1</sup>, a purification of 400-fold relative to the initial supernatant. The specific activity of peak II was 8.8 µmol.(mg protein)<sup>-1</sup>.min<sup>-1</sup>, a purification of 700-fold relative to the initial supernatant (Table 1).

Table 1 shows the purification of soluble starch synthase from developing potato tubers of Desirée. Fractions were prepared as described above. The values shown in the table are from a typical purification.

TABLE 1

FRACTION	TOTAL ACTIVITY (µmol glucose incorporated min <sup>-1</sup> )	ACTIVITY RECOVERED (%)	TOTAL PROTEIN (mg)	SPECIFIC ACTIVITY (µmol glucose incorporated min <sup>-1</sup> . mg protein <sup>-1</sup> )
Initial Supernatant	28.9	100	2210.6	0.013
0 to 40% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	17.1	61.0	1018.6	0.017
DEAE-Sepharose	7.51	26.8	45.1	0.166
Blue-Sepharose	4.59	16.4	10.4	0.441
Peak I				
Mono Q (pH 7.5)	0.95	3.4	1.70	0.56
Cyclohexa-amylose	0.53	1.9	0.20	2.65



TABLE 1 (continued)

FRACTION	TOTAL ACTIVITY ( $\mu\text{mol}$ glucose incorporated $\text{min}^{-1}$ )	ACTIVITY RECOVERED (%)	TOTAL PROTEIN (mg)	SPECIFIC ACTIVITY ( $\mu\text{mol}$ glucose incorporated $\text{min}^{-1}$ , mg protein $^{-1}$ )
Peak I				
Mono Q (pH 8.0)	0.15	0.5	0.03	5.13
Peak II				
Mono Q (pH 7.5)	2.45	8.8	2.90	0.85
Cyclohexa-amylose	2.27	8.1	0.40	5.67
Mono Q (pH 8.0)	0.26	0.9	0.03	0.84

TABLE 2

INCUBATION	INHIBITION OF STARCH SYNTHASE ACTIVITY (%)
Pre-immune serum	$0.3 \pm 0.9$
Antiserum to potato SSS	$74 \pm 4$
Antiserum to pea GBSS II	$9 \pm 4$
Antiserum to potato SSS + pea GBSS II	$80 \pm 8$

Table 2 shows the immunoprecipitation of starch synthase activity in soluble extract from developing Desiree tubers. Soluble extract was incubated in the presence of antiserum (1/10 dilution of rat antiserum; or 1/5 dilution of the immunoglobulin fraction of rabbit serum to GBSS II from pea embryo; or 1/10 dilution of rat antiserum plus 1/5 dilution of the immunoglobulin fraction of rabbit serum to GBSS II), as described in Material and Methods. After centrifugation the supernatant was assayed for starch synthase activity. Values are percentage inhibition relative to controls in which BSA at  $20 \text{ g.l}^{-1}$  in PBS was substituted for serum. The values are the mean of four experiments  $\pm$  standard error.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of the fractions from the second Mono Q<sup>TM</sup> column for peak I showed that the distribution of a protein of 120 kDa matched the distribution of the starch synthase activity (Figure 2). Further chromatography on Mono Q<sup>TM</sup> did not eliminate contaminating proteins. SDS-PAGE of the fractions from the final Mono Q<sup>TM</sup> column for peak II showed that the distribution of the major protein of 110 kDa matched the starch synthase activity (Figure 2).

Antibodies raised to the 59 kDa starch-granule-bound protein (the GBSS I isoform) from pea embryo did not recognise any proteins from either peak I or peak II. Antibodies raised to the 77 kDa GBSS II from pea embryo very weakly recognised the 120 and 110 kDa proteins from peak I and II respectively (data not shown).

#### Preparation of antibody.

In order to obtain sufficient protein for preparation of an antibody, peaks I and II from mature Estima tubers were combined and purified together in large-scale preparations (referred to as "soluble starch synthase", SSS). Both the 120- and 110 kDa proteins were excised and eluted from gels of the purified preparations and were injected into the same rat.

The antiserum to the SSS was used to probe blots of extracts from mature Estima and developing Desiree tubers (Figure 3). On all of the immunoblots, the pre-immune serum did not cross-react with any of the proteins. On immunoblots of the gels of the purified preparation of soluble starch synthase from mature Estima tubers, the antiserum recognised strongly the two proteins to which it was raised. The antiserum also recognised a minor protein of 140 kDa. On immunoblots of gels of partially purified soluble starch synthase from mature Estima tubers, the antiserum recognised proteins of 140 kDa and 120 kDa. On immunoblots of gels of starch-granule-bound proteins from mature Estima tubers, the antiserum recognised a protein of 140 kDa. There were some faint indications that a 120 kDa protein on the starch was also recognised. A protein of 140 kDa was recognised by the antiserum both in the soluble extracts and on starch granules of developing tubers of Desiree. The 120 kDa protein was very weakly detectable in soluble extracts from these tubers, which also contained a lower molecular weight protein (approximately 100 kDa) recognised

by the antiserum. This protein is not GBSS II since the rat antiserum did not recognise GBSS II on starch granules (data not shown).

#### Immunoprecipitation of starch synthase activity.

To discover whether the proteins recognised by the antiserum to SSS represent the major soluble starch synthases, the antiserum was used in immunoprecipitation experiments with soluble extracts from developing Desiree tubers.

Incubation of soluble extract with pre-immune serum from the rat did not affect soluble starch synthase activity, but the antiserum to SSS precipitated starch synthase activity (Figure 4). The maximum inhibition of starch synthase activity was approximately 75 % which was achieved by incubating with volumes greater than 2  $\mu$ l of antiserum. A small proportion of the remaining starch synthase activity can be accounted for by GBSS II (Table 2). When soluble extract is incubated with antiserum raised to GBSS II from pea embryo (which recognises GBSS II in potato, Edwards *et al.*, 1995), approximately 9% of the starch synthase activity is inhibited. When the potato extract is mixed with both antibodies, the starch synthase activity is reduced by approximately 80%.

Native polyacrylamide gel electrophoresis of soluble extracts of developing Desiree tubers revealed two major groups of bands which had starch synthase activity (Figure 5). We have previously shown through antisense and immunoprecipitation experiments that the lower group of bands is attributable to GBSS II. Tubers in which GBSS II protein has been severely reduced by antisense transformation lack the lower group of bands. When the soluble extract was immunoprecipitated with antiserum to GBSS II from pea embryo and the supernatant subjected to native PAGE, the lower bands were missing (Edwards *et al.*, 1995 cited previously). Immunoprecipitation of soluble extract from developing Desiree tubers with rat antiserum to SSS shows that the upper group of bands is attributable to these starch synthases. When the supernatant from the immunoprecipitation experiment was subjected to native PAGE, the upper group of bands was missing but the lower group was unaffected. The pre-immune serum from rat had no effect on the bands of starch synthase activity.

#### DISCUSSION

The inventors have purified two proteins with starch synthase activity from the soluble fraction from mature Estima tubers, with molecular weights of 110 and 120 kDa respectively. Immunoblots show that the antiserum raised to these purified proteins (soluble starch synthases, SSS) recognises the proteins to which it was raised, and that it also recognises a higher molecular weight protein of 140 kDa in the purified preparation (Figure 3). The 140 kDa protein is in soluble extracts and on starch granules of both mature Estima and developing Desiree tubers, whereas the 120 kDa protein is either barely or not detectable in tubers, and the 110 kDa protein is not detectable at all. This strongly suggests that the two starch synthase proteins to which antibodies have been raised may both be active breakdown products of the larger 140 kDa protein, although the 140 kDa polypeptide might simply be an immunologically cross-reactive entity. Although most of the breakdown undoubtedly occurs during purification (despite the purification being carried out at 4°C and with the inclusion of PVPP and protease inhibitors), some of the breakdown may also occur *in vivo*. Breakdown of enzymes *in vivo* has been observed during the purification of starch branching enzyme from potato tubers. Using fresh harvested tubers for the purification resulted in a predominately high molecular weight starch branching enzyme being isolated, but when stored tubers were used, a wide range of molecular weight proteins were isolated (Blennow & Johansson, 1991 *Phytochem.* 30, 437-444).

The fact that the antiserum to SSS recognises the 140 kDa protein in both Estima and Desiree tubers suggests that there is no difference between the two cultivars in their major starch synthases, and vindicates the use of these two different cultivars in the work reported herein. The occurrence of a 100 kDa protein antigenically related to the 140 kDa protein in Desiree tubers is interesting, and at present it is not known what that protein may be. Its absence from Estima tubers could reflect the fact that these cultivars were stored rather than developing tubers, or could represent a difference between cultivars.

Specific activities of the purified soluble starch synthases from potato tuber are comparable with or greater than these of soluble starch synthases from other storage organs. Purification to homogeneity of isoforms of soluble starch synthase resulted in specific activities of 16  $\mu$ mol.(mg protein)<sup>-1</sup>.min<sup>-1</sup> from pea embryo (Denyer & Smith 1992 cited previously), 14  $\mu$ mol.(mg protein)<sup>-1</sup>.min<sup>-1</sup> from wheat (Denyer *et al.*, 1995 cited previously) and 9  $\mu$ mol.(mg protein)<sup>-1</sup>.min<sup>-1</sup> from maize (Mu *et al.* (1994) *Plant J.* 6, 151-159). The specific activity of the soluble starch synthase reported in this application is 7- to 300-fold higher than that of the partial purifications of soluble starch synthase activity from potato tuber reported by Hawker *et al.*, 1972 *Phytochem.* 11, 1278-1293: 0.64  $\mu$ mol.(mg protein)<sup>-1</sup>.min<sup>-1</sup>, Baba *et al.* (1990) *Phytochem.* 29, 719-723: 0.03  $\mu$ mol.(mg protein)<sup>-1</sup>.min<sup>-1</sup> and Ponstein (1.35 and 0.91  $\mu$ mol.(mg protein)<sup>-1</sup>.min<sup>-1</sup>).

The immunoprecipitation experiments also suggest that the purified proteins are the major soluble starch synthases in potato tuber, or are products directly derived from such synthases. The antiserum raised against the soluble starch

synthase from potato precipitates 75 % of the total soluble starch synthase activity in crude extract (Figure 5). The remainder of the activity is partly due to GBSS II (Table 2), but the possibility of further minor isoforms cannot be ruled out.

The purified soluble starch synthase is likely to represent a novel class of starch synthase. It is not related to the major soluble starch synthase in pea embryo (GBSS II), which is clearly related to the minor, soluble 92 kDa GBSS II in potato. The soluble starch synthase is only very weakly recognised by the antibody raised to GBSS II from pea. It is not related to the GBSS I proteins either: the starch synthase from potato tuber is not recognised by the antibody raised to GBSS I from pea embryo. These results reinforce the view that storage organs differ profoundly in the nature and number of active isoforms of starch synthase (Smith *et al.*, 1995 Plant Physiol. 107, 1; Edwards *et al.*, and Denyer *et al.*, both cited previously).

## Example 2

### ISOLATION OF A cDNA CLONE FOR SOLUBLE STARCH SYNTHASE FROM POTATO TUBERS

The antiserum raised to the purified starch synthase protein from Estima tubers was used for immunoscreening of a  $\lambda$ gt 11 library (provided by C Grierson, John Innes Centre, Norwich) containing cDNA inserts with *Eco*RI linkers, constructed from developing Estima tuber poly(A) RNA.

Approximately  $1.5 \times 10^6$  plaque-forming units were probed with the antiserum at a dilution of 1/1000. The second antibody was an anti-rat immunoglobulin linked to horseradish peroxidase (Amersham International, Amersham, UK). Two positive clones were isolated. These were both 1.1 kb in length and contained poly(A) tracts at their 3' ends. One of these was cloned into the *Eco*RI site of pBluescript SK+ to give plasmid pRAT2. A 5' *Eco*RI-*ECORV* fragment from this clone was used as a probe on the  $\lambda$ gt11 library. Filters were washed in 0.1 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.5 g L<sup>-1</sup> SDS at 65°C. Seven clones of 1.3, 1.53, 1.75, 1.88, 2.15, 2.21, and 2.4 kb were isolated. The longest clone was subcloned as an *Eco*RI fragment into pBluescript SK+ to give plasmid pRAT20. A 600-bp 5' fragment from pRAT20 was used to probe a random primed  $\lambda$ gt11 library prepared from cDNA of developing tubers. Three positive clones were isolated. The longest was 2.3 kb and was subcloned as an *Eco*RI fragment into pBluescript SK+ to give pRAT24.

The 2.3 and 2.4kb partial clones overlapped. The full-length composite cDNA was 4.127kb. The DNA sequence of the full length cDNA, and the predicted polypeptide sequence, are shown in Figure 6. DNA sequences were determined according to Sanger *et al.* (1977) by using Sequenase™ (United States Biochemical). Sequence data were analysed using the Genetics Computer Group (Madison, WI) computer program (Devereux *et al.* 1984 Nucl. Acids Res. 12, 387-395).

To check the identity of the cDNA, the amino acid sequence it predicted was compared with amino acid sequences of two peptides obtained by digestion with endoproteinase Lys-C of the 110-kD protein purified from tubers of cultivar Estima. The peptide sequences FIPIPYTSENVVEGK (Seq. ID No. 1) and HIPVFGG (Seq. ID No. 2) corresponded precisely to predicted sequences from the clone. Attempts to obtain N-terminal amino acid sequence of the purified proteins for comparison with the sequence predicted from the cDNA clone were unsuccessful.

On RNA gel blots of poly(A)<sup>+</sup>RNA from developing tubers, a partial cDNA clone recognised a single transcript of ~4 kb. This size is considerably greater than those of the transcripts for GBSSI and GBSSII and is consistent with the transcript encoding a protein in the range of 110 to 140 kD.

The deduced amino acid sequence of the soluble starch synthase revealed a protein of 1230 amino acids and a predicted size of 139 kD (Figure 6). At the N terminus was a sequence of ~60 amino acids rich in serine and basic residues and low in acidic residues, which is typical of a chloroplast transit peptide. Based on the consensus of Gavel and von Heinje (1990 FEBS Lett. 261, 455-458), the most likely cleavage site would be between amino acids 60 (Cys) and 61 (Ala), because the serine-rich region ends before this point. Cleavage in this region would give a mature protein of ~132 kD. The structure is somewhat similar to that of GBSSII in that it contains a C-terminal region homologous with starch synthases and bacterial glycogen synthases and an N-terminal extension. The N-terminal extension shows little sequence similarity to the N-terminal extensions of GBSSII from pea or potato (in turn, they show little similarity to each other; Edwards *et al.*, (1995) Plant J. 8, 283-294) or to any other sequence in the data bases. The N-terminal domain resembles those of pea and potato GBSSII in that it shows considerable predicted flexibility (Chou-Fasman algorithm; see Dry *et al.*, (1992) Plant J. 2, 193-202); all these extensions may therefore serve similar roles. At the C-terminal end of the N-terminal extension of the soluble starch synthase are two proline residues; multiple proline residues have been noted previously at the C-terminal ends of N-terminal extensions of both starch synthases and starch-branching enzymes (Dry *et al.*, (1992) Plant J. 2, 193-202; Burton *et al.*, 1995).

The roles of these N-terminal extensions are not known, but it seems likely that they are involved in determining properties such as interaction with starch polymers rather than contributing to basic catalytic properties. The C-terminal region from amino acid 780 to the end shows greatest similarity to glycogen synthases from bacteria, although there

is also similarity to other starch synthases from plants. The KTGG motif close to the N terminus of this region beginning with position 794 is conserved (KVGGL). This domain is thought to be involved in ADP/ADP-glucose binding (Furukawa *et al.*, 1990 J. Biol. Chem. 265, 2086-2090). Interestingly, a second domain with a similar structure is also conserved in the C termini of all bacterial glycogen synthases and starch synthases (including the motif beginning at position 1143, T/V GGLXDT I/V); this may represent a second domain involved in ADP/ADP-glucose binding. The whole region around this second domain is widely conserved among  $\alpha$ -1,4-glucosyltransferases, indicating close involvement with the catalytic process.

Over the rest of the soluble starch synthase protein, there are several other domains showing conservation between different starch synthases. However, it also shows some notable gaps in its sequence when aligned with GBSSI and GBSII, for example, between amino acids 828 to 829 (13 amino acids), 894 to 895 (10 amino acids), and 944 to 945 (35 amino acids). These regions may confer specific properties on GBSSI and GBSII compared with the soluble synthase.

### Example 3

#### POTATO TRANSFORMATION

Binary vectors containing a partial cDNA for soluble starch synthase ("SSS") in the antisense orientation, under the control of a) the double 35S promoter or b) the patatin promoter have been constructed. The 2 x 35S construct is detailed below.

#### Construction of Antisense Binary Vector

The 1.1-kb *Pst*-*EcoRV* fragment from pRAT2 encoding the 3' end of the soluble starch synthase was subcloned in an antisense orientation between the cauliflower mosaic virus double 35S promoter and cauliflower mosaic virus terminator (*Pst*I-*Sma*I) in pJIT60 (Guerineau and Mullineaux, 1993 "Plant transformation and expression vectors". In Plant Molecular Biology Labfax R.R.D. Croy, Ed. (Oxford, UK BIOS Scientific Publishers) p121-148), producing pRAT3. The *Xho*I-partial *Sst*I fragment from pRAT3, encompassing the promoter, antisense cDNA, and terminator, was ligated between the *Sal*I-*Sst*I sites of the plant transformation vector pBIN19 (Bevan, 1984 Nucl. Acids Res. 12, 8711-8721), resulting in plasmid pRAT4. This plasmid is illustrated schematically in Figure 7, which Figure also shows the plasmid pPATRAT comprising the patatin promoter.

#### Transformation of Potato

Binary plasmid pRAT4 was introduced into *Agrobacterium tumefaciens* by the freeze-thaw method of An *et al.*, (1988 Binary Vectors. In Plant Molecular Biology Manual A3. Eds, Gelvin S.B. and Schilperoort R.A. ppl-19). Preparation of *Agrobacterium* inoculum carrying the antisense construct, inoculation of tuber discs of potato cultivar Desiree, regeneration of shoots, and rooting of shoots were as described by Edwards *et al.* (1995 Plant J. 8, 283-294).

Thirteen independently transformed plants and four independent control plants (transformed with the vector alone) were transferred to a soil-based compost and allowed to develop tubers. The presence of the SSS antisense construct was confirmed by DNA gel blotting (data not shown). Six of the transgenic plants had levels of SSS transcript indistinguishable from those of the control plants on RNA gel blots. However, seven independent transformants (named 1, 2, 9, 18, 19, 25 and 26) had strongly reduced or undetectable levels of SSS transcript. The loss or reduction of detectable transcript was specific for SSS, and there was little variation in the level of transcript for GBSSI among the plants studied (data shown in Marshall *et al.*, 1996 The Plant Cell 8, 1121-1135).

Tubers of the transformants with unaltered levels of SSS transcript had soluble starch synthase activities that were indistinguishable from those of the control plants and from values typical of those obtained from developing Desiree tubers in general (Edwards *et al.* (1995) Plant J. 8, 283-294 1995). Tubers of the seven transformants with reduced or undetectable levels of SSS transcript had significantly reduced activities, and three plants displayed activities that were 30% or less of the average value of the control plants. Table 3 shows that the observed reductions in soluble starch synthase activity were reproducible from one tuber to another. They were also reproducible through tuber development.

**Table 3.** Effects of Reduced Activity of SSS on Soluble and Granule-Bound Starch Synthase Activity and Amylose Content of Starch.

Plant <sup>a</sup>	Soluble Activity <sup>b</sup> (nmol min <sup>-1</sup> g <sup>-1</sup> Fresh Weight)	Granule-Bound Activity <sup>c</sup> (nmol min <sup>-1</sup> g <sup>-1</sup> Fresh Weight)	Amylose Content <sup>d</sup> (% Total Starch)
1	ND <sup>e</sup>	ND	27.8
2	ND	ND	29.5, 29.8
9	18.3 ± 3.9 (4)	118	28.6
18	23.6 ± 6.7 (3)	97	29.3
25	29.5 ± 3.6 (4)	113	27.3
26	33.3 ± 8.3 (3)	80	30.1
Control	98.4 ± 4.9 (9)	106 ± 12	26.4, 28.9
Desiree	ND	ND	27.8, 29.2

<sup>a</sup> Plant numbers refer to individual transgenic plants with reduced SSS activity. Tubers are from a single plant, except for the control line, in which three different plants (each an independent, control transformant) were used.

<sup>b</sup> Soluble activity was measured by using duplicate samples from tubers of 12 to 70 g fresh weight harvested at intervals during plant development. Values are the means ± SE of measurements made with the number of tubers given within parentheses.

<sup>c</sup> Granule-bound activities are the means of measurements made by using duplicate samples from a single tuber (12 to 70 g fresh weight) harvested at maturity.

<sup>d</sup> Amylose content was measured by using starch extracted from two or three tubers per mature plant. Values are the means of measurements made with two separate samples taken from the bulk starch preparations: two values are given when independent starch preparations were used. Wild-type Desiree plants used for these measurements were grown in the same greenhouse at the same time as the transgenic

plants.

<sup>c</sup> ND, not determined.

### Reductions in Starch Synthase Activity Are Specifically Due to Loss of SSS

To discover whether the reductions in activity were specifically attributable to loss of SSS, two sorts of experiments were undertaken. First, isoforms were visualised on native gels of crude, soluble extracts of transformed tubers. The group of bands attributable to SSS was present in extracts from control plants and from all six of the transformants with soluble starch synthase activities comparable with control activities. It was absent from extracts of all seven transformants with reduced starch synthase activities. Other groups of bands on the gels, including those attributable to GBSSII, were present in all extracts (data shown in Marshall *et al.*, 1996).

Second, crude, soluble extracts from a plant with strongly reduced activity were incubated with the antiserum raised against SSS. The antiserum inhibited activity by 16%, compared with 75 % inhibition in extracts of untransformed tubers of cultivar Desiree (Table 2).

Loss of starch synthase activity from the soluble fraction in transgenic tubers was accompanied by dramatic reductions in the amount of the 140-kD protein recognised by the antiserum in soluble and granule-bound fractions of the tuber. The protein was not detected, or detected only very weakly relative to controls, on immunoblots of these fractions from tubers of the six transgenic lines with the largest reductions in starch synthase activity. In contrast, the soluble protein of 105 kD also recognised by the antiserum was present in equal amounts in all lines examined (data shown in Marshall *et al.*, 1996).

### Reduction in SSS Activity Alters Granule Shape but Has Little Effect on Starch and Amylose Content

Tubers of the seven transformants with reduced activities of soluble starch synthase had starch granules with strikingly altered morphology. Two types of granule were present: simple granules with deep, often T-shaped cracks centered on the hilum, and granules that appeared to be large clusters of tiny, spherical granules. A range of different sizes of both types of granule was present in tubers at various developmental stages (data not shown).

In spite of the alteration in granule morphology, tubers of transformants with reduced activity of SSS were indistinguishable from control tubers with respect to total starch content. This was true of both developing tubers and tubers of mature plants on which the haulm was senescent. The starch of these plants also displayed no significant alteration in amylose content (Table 3).

### Reduction in SSS Activity Does Not Affect Other Isoforms of Starch Synthase

It was thought possible that the reduction in SSS in transformed tubers may have secondary effects on other isoforms of starch synthase. Any alterations in other isoforms could seriously affect deductions about the importance and role of SSS and might prevent alteration of starch properties in transformed plants. Effects of the reduction in SSS on GBSSI were assessed by measuring granule-bound starch synthase activity in crude extracts of tubers and examining gels of granule-bound proteins. There was no difference in granule-bound activity between control plants and those in which soluble starch synthase activity was reduced (Table 3). More than 95 % of the starch synthase activity of intact starch granules of wild-type potatoes is attributable to GBSSI (Edwards *et al.* (1995) Plant J. 8, 283-294 1995). Reductions in SSS also had no obvious effect on the amount of GBSSI protein bound to starch granules (data not shown).

Effects of reductions in SSS on GBSSII were assessed in three ways. First, amounts of GBSSII protein in the soluble and granule-bound fractions of the tuber were visualised by immunoblotting. There were no obvious differences between control plants and those in which SSS was reduced.

Second, as described above, GBSSII was visualised on native gels of crude, soluble extracts stained for starch synthase activity. Again, there were no marked or consistent differences between control plants and those in which SSS was reduced.

Third, immunoprecipitation experiments were used to assess the proportion of the residual activity attributable to GBSSII in tubers in which SSS was reduced. The antiserum raised against GBSSII of pea embryos, which recognises GBSSII of potatoes (Edwards *et al.* 1995 cited above), inhibited ~40% of the activity in tubers in which soluble starch synthase activity was reduced by ~80% (line 9) compared with 9% in control and wild-type tubers (Table 2). Using these figures and starch synthase activities from Table 3, the activity attributable to GBSSII is 7.3 nmol min<sup>-1</sup>g<sup>-1</sup> fresh

weight in line 9 and  $8.8 \text{ nmol min}^{-1} \text{g}^{-1}$  fresh weight in control tubers. This indicates that the reduction in SSS has little effect on the soluble activity of GBSSII.

#### **Example 4**

##### **Detailed analysis of starch from tubers obtained from transformed Potato plants**

Despite the results of crude analysis described in Example 3, indicating that the starch from transformed plants was essentially unaltered, it was decided to perform more detailed analysis of the starch, by Differential Scanning Calorimetry and Viscoamylograph. Analysis was performed as described in WO 95/26407 and WO 96/34968.

Surprisingly it was found that certain physical properties of the starch were consistently significantly altered. In particular, it was found that the viscosity onset temperature was significantly reduced compared to starch obtained from equivalent control plants which did not contain the SSS antisense construct. The results are shown in Table 4.

Starch from AS major soluble starch synthase tubers

TABLE 4

Plant line	Plant n°	DSC			RVA	SSS activity	Apparent amylose*	Granule morphology
		Peak (°C)	Onset (°C)	Delta H (J/g)	Onset (°C)	(nmol/min/ mg of tuber)	(% w/w)	Comments
Rat 4.1		65.8	61.7	16.1	63.4		27.82	unusual granules: some compound, some with large cracks
Rat 4.2	1	65.5	61.6	16.0	62.0		29.76	unusual granules (as Rat 4.1)
	2	63.4	59.4	17.0	61.5		29.45	DSC endotherm double peak
		63.6	59.6	16.7				
Désirée (control)	1	64.0	60.1	16.9				
		63.1	59.3	17.4	65.6		29.16	
		67.6	64.4	17.5				
	2	67.5	64.4	17.2				
		67.3	64.6	16.9	65.6		27.80	
		67.3	64.7	17.5				
Rat 4.9		57.4	53.2	14.5		18.3	28.60	unusual granules (as Rat 4.1)
		57.4	53.2	14.0				
Rat 4.18		58.6	54.3	(14.5)		23	29.31	unusual granules (as Rat 4.1), but smaller than controls. DSC endotherm broad
		58.5	54.3	(16.0)				unusual granules (as Rat 4.1)
Rat 4.25		59.0	54.3	14.8		24	27.32	
		59.0	54.2	14.9				
Rat 4.26		(59.6)	54.9	(16.2)		30	30.10	unusual granules (as Rat 4.1)
		(59.8)	54.9	(16.9)				DSC endotherm double peak
Luc 1 (control)		63.7	61.0	17.1		110	26.43	
		63.6	61.3	17.6				
Luc 6 (control)		64.6	62.0	17.9		116	28.85	
		64.5	62.0	17.3				

DSC      Differential scanning calorimetry      SSS activity      Soluble starch synthase activity

Onset: endotherm onset temperature  
Peak: endotherm peak temperature  
Delta H: endotherm enthalpy

RVA      Rapid visco analyser  
Onset: viscosity onset temperature



## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

(A) NAME: National Starch and Chemical Investment  
Holding Corporation

(B) STREET: Suite 27, 501 Silverside Road

(C) CITY: Wilmington

(D) STATE: Delaware

(E) COUNTRY: U.S.A.

(F) POSTAL CODE (ZIP): DE 19809

(ii) TITLE OF INVENTION: Improvements in or Relating to Soluble  
Starch Synthase

(iii) NUMBER OF SEQUENCES: 4

## (iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0. Version #1.30 (EPO)

## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Phe Ile Pro Ile Pro Tyr Thr Ser Glu Asn Val Val Glu Gly Lys  
1 5 10 15

## (2) INFORMATION FOR SEQ ID NO: 2:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 7 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

His Ile Pro Val Phe Gly Gly  
1 5

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 4127 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Solanum tuberosum
- (B) STRAIN: Desiree
- (F) TISSUE TYPE: tuber

(vii) IMMEDIATE SOURCE:

- (A) LIBRARY: lambda gt11

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION:143..3835

(ix) FEATURE:

- (A) NAME/KEY: sig\_peptide
- (B) LOCATION:143..322

(ix) FEATURE:

- (A) NAME/KEY: mat\_peptide
- (B) LOCATION:323..3832

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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GAATTCGCGG CCGCAGATAG TGTGTTGATG AAGGAGAAGA GAGATATTTC ACATGGGATG  60
TTCTATTTGA TTCTGTGGTG AACAAAGAGTT TTACAAAGAA CATTCTTTT TCTTTTTTCC 120
TTGGTTCTTG TGTGGGTCAG CC ATG GAT GTT CCA TTT CCA CTG CAT AGA TCA  172
                               Met Asp Val Pro Phe Pro Leu His Arg Ser
                               -60                               -55

TTG AGT TGC ACA AGT GTC TCC AAT GCA ATA ACC CAC CTC AAG ATC AAA  220
Leu Ser Cys Thr Ser Val Ser Asn Ala Ile Thr His Leu Lys Ile Lys
-50                               -45                               -40                               -35

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	CCT	ATT	CTT	GGG	TTT	GTC	TCT	CAT	GGA	ACC	ACA	AGT	CTA	TCA	GTA	CAA	268
	Pro	Ile	Leu	Gly	Phe	Val	Ser	His	Gly	Thr	Thr	Ser	Leu	Ser	Val	Gln	
				-30					-25						-20		
5	TCT	TCT	TCA	TGG	AGG	AAG	GAT	GGA	ATG	GTT	ACT	GGG	GTT	TCA	TTT	TCC	316
	Ser	Ser	Ser	Trp	Arg	Lys	Asp	Gly	Met	Val	Thr	Gly	Val	Ser	Phe	Ser	
				-15				-10					-5				
10	ATT	TGT	GCA	AAT	TTC	TCG	GGA	AGA	AGA	CGG	AGA	AAA	GTT	TCA	ACT	CCT	364
	Ile	Cys	Ala	Asn	Phe	Ser	Gly	Arg	Arg	Arg	Arg	Lys	Val	Ser	Thr	Pro	
			1				5					10					
15	AGG	AGT	CAA	GGC	TCT	TCA	CCT	AAG	GGG	TTT	GTG	CCA	AGG	AAG	CCC	TCA	412
	Arg	Ser	Gln	Gly	Ser	Ser	Pro	Lys	Gly	Phe	Val	Pro	Arg	Lys	Pro	Ser	
	15					20				25					30		
20	GGG	ATG	AGC	ACG	CAA	AGA	AAG	GTT	CAG	AAG	AGC	AAT	GGT	GAT	AAA	GAA	460
	Gly	Met	Ser	Thr	Gln	Arg	Lys	Val	Gln	Lys	Ser	Asn	Gly	Asp	Lys	Glu	
				35					40					45			
25	AGT	AAA	AGT	ACT	TCA	ACA	TCT	AAA	GAA	TCT	GAA	ATT	TCC	AAC	CAG	AAG	508
	Ser	Lys	Ser	Thr	Ser	Thr	Ser	Lys	Glu	Ser	Glu	Ile	Ser	Asn	Gln	Lys	
				50				55					60				
30	ACG	GTT	GAA	GCA	AGA	GTT	GAA	ACT	AGT	GAC	GAT	GAC	ACT	AAA	GGA	GTG	556
	Thr	Val	Glu	Ala	Arg	Val	Glu	Thr	Ser	Asp	Asp	Asp	Thr	Lys	Gly	Val	
			65				70					75					
35	GTG	AGG	GAC	CAC	AAG	TTT	CTG	GAG	GAT	GAG	GAT	GAA	ATC	AAT	GGT	TCT	604
	Val	Arg	Asp	His	Lys	Phe	Leu	Glu	Asp	Glu	Asp	Glu	Ile	Asn	Gly	Ser	
		80				85						90					
40	ACT	AAA	TCA	ATA	AGT	ATG	TCA	CCT	GTT	CGT	GTA	TCA	TCT	CAA	TTT	GTT	652
	Thr	Lys	Ser	Ile	Ser	Met	Ser	Pro	Val	Arg	Val	Ser	Ser	Gln	Phe	Val	
		95				100				105					110		
45	GAA	AGT	GAA	GAA	ACT	GGT	GGT	GAT	GAC	AAG	GAT	GCT	GTA	AAG	TTA	AAC	700
	Glu	Ser	Glu	Glu	Thr	Gly	Gly	Asp	Asp	Lys	Asp	Ala	Val	Lys	Leu	Asn	
				115					120					125			
50	AAA	TCA	AAG	AGA	TCG	GAA	GAG	AGT	GGT	TTT	ATA	ATT	GAT	TCT	GTA	ATA	748
	Lys	Ser	Lys	Arg	Ser	Glu	Glu	Ser	Gly	Phe	Ile	Ile	Asp	Ser	Val	Ile	
			130					135					140				
55	AGA	GAA	CAA	AGT	GGA	TCT	CAG	GGG	GAA	ACT	AAT	GCC	AGT	AGC	AAG	GGA	796
	Arg	Glu	Gln	Ser	Gly	Ser	Gln	Gly	Glu	Thr	Asn	Ala	Ser	Ser	Lys	Gly	
			145				150					155					
60	AGC	CAT	GCT	GTG	GGT	ACA	AAA	CTT	TAT	GAG	ATA	TTG	CAG	GTG	GAT	GTT	844
	Ser	His	Ala	Val	Gly	Thr	Lys	Leu	Tyr	Glu	Ile	Leu	Gln	Val	Asp	Val	
		160				165						170					
65	GAG	CCA	CAA	CAA	TTG	AAA	GAA	AAT	AAT	GCT	GGG	AAT	GTT	GAA	TAC	AAA	892
	Glu	Pro	Gln	Gln	Leu	Lys	Glu	Asn	Asn	Ala	Gly	Asn	Val	Glu	Tyr	Lys	
	175					180					185					190	

EP 0 779 363 A2

	GGA	CCT	GTA	GCA	AGT	AAG	CTA	TTG	GAA	ATT	ACT	AAG	GCT	AGT	GAT	GTG	940
	Gly	Pro	Val	Ala	Ser	Lys	Leu	Leu	Glu	Ile	Thr	Lys	Ala	Ser	Asp	Val	
					195					200					205		
5	GAA	CAC	ACT	GAA	AGC	AAT	GAG	ATT	GAT	GAC	TTA	GAC	ACT	AAT	AGT	TTC	988
	Glu	His	Thr	Glu	Ser	Asn	Glu	Ile	Asp	Asp	Leu	Asp	Thr	Asn	Ser	Phe	
				210					215					220			
10	TTT	AAA	TCA	GAT	TTA	ATT	GAA	GAG	GAT	GAG	CCA	TTA	GCT	GCA	GGA	ACA	1036
	Phe	Lys	Ser	Asp	Leu	Ile	Glu	Glu	Asp	Glu	Pro	Leu	Ala	Ala	Gly	Thr	
			225					230					235				
15	GTG	GAG	ACT	GGA	GAT	TCT	TCT	CTA	AAC	TTA	AGA	TTG	GAG	ATG	GAA	GCA	1084
	Val	Glu	Thr	Gly	Asp	Ser	Ser	Leu	Asn	Leu	Arg	Leu	Glu	Met	Glu	Ala	
		240					245					250					
20	AAT	CTA	CGT	AGG	CAG	GCT	ATA	GAA	AGG	CTT	GCC	GAG	GAA	AAT	TTA	TTG	1132
	Asn	Leu	Arg	Arg	Gln	Ala	Ile	Glu	Arg	Leu	Ala	Glu	Glu	Asn	Leu	Leu	
	255				260						265					270	
25	CAA	GGG	ATC	AGA	TTA	TTT	TGT	TTT	CCA	GAG	GTT	GTA	AAA	CCT	GAT	GAA	1180
	Gln	Gly	Ile	Arg	Leu	Phe	Cys	Phe	Pro	Glu	Val	Val	Lys	Pro	Asp	Glu	
					275				280						285		
30	GAT	GTC	GAG	ATA	TTT	CTT	AAC	AGA	GGT	CTT	TCC	ACT	TTG	AAG	AAT	GAG	1228
	Asp	Val	Glu	Ile	Phe	Leu	Asn	Arg	Gly	Leu	Ser	Thr	Leu	Lys	Asn	Glu	
				290				295					300				
35	TCT	GAT	GTC	TTG	ATT	ATG	GGA	GCT	TTT	AAT	GAG	TGG	CGC	TAT	AGG	TCT	1276
	Ser	Asp	Val	Leu	Ile	Met	Gly	Ala	Phe	Asn	Glu	Trp	Arg	Tyr	Arg	Ser	
			305				310						315				
40	TTT	ACT	ACA	AGG	CTA	ACT	GAG	ACT	CAT	CTC	AAT	GGA	GAT	TGG	TGG	TCT	1324
	Phe	Thr	Thr	Arg	Leu	Thr	Glu	Thr	His	Leu	Asn	Gly	Asp	Trp	Trp	Ser	
		320					325					330					
45	TGC	AAG	ATC	CAT	GTT	CCC	AAG	GAA	GCA	TAC	AGG	GCT	GAT	TTT	GTG	TTT	1372
	Cys	Lys	Ile	His	Val	Pro	Lys	Glu	Ala	Tyr	Arg	Ala	Asp	Phe	Val	Phe	
	335				340					345					350		
50	TTT	AAT	GGA	CAA	GAT	GTC	TAT	GAC	AAC	AAT	GAT	GGA	AAT	GAC	TTC	AGT	1420
	Phe	Asn	Gly	Gln	Asp	Val	Tyr	Asp	Asn	Asn	Asp	Gly	Asn	Asp	Phe	Ser	
				355					360						365		
55	ATA	ACT	GTG	AAA	GGT	GGT	ATG	CAA	ATC	ATT	GAC	TTT	GAA	AAT	TTC	TTG	1468
	Ile	Thr	Val	Lys	Gly	Gly	Met	Gln	Ile	Ile	Asp	Phe	Glu	Asn	Phe	Leu	
				370				375					380				
60	CTT	GAG	GAG	AAA	TGG	AGA	GAA	CAG	GAG	AAA	CTT	GCT	AAA	GAA	CAA	GCT	1516
	Leu	Glu	Glu	Lys	Trp	Arg	Glu	Gln	Glu	Lys	Leu	Ala	Lys	Glu	Gln	Ala	
			385					390					395				
65	GAA	AGA	GAA	AGA	CTA	GCA	GAA	GAA	CAA	AGA	CGA	ATA	GAA	GCA	GAG	AAA	1564
	Glu	Arg	Glu	Arg	Leu	Ala	Glu	Glu	Gln	Arg	Arg	Ile	Glu	Ala	Glu	Lys	
		400					405					410					

EP 0 779 363 A2

	GCT	GAA	ATT	GAA	GCT	GAC	AGA	GCA	CAA	GCA	AAG	GAA	GAG	GCT	GCA	AAG	1612
	Ala	Glu	Ile	Glu	Ala	Asp	Arg	Ala	Gln	Ala	Lys	Glu	Glu	Ala	Ala	Lys	
	415					420					425					430	
5	AAA	AAG	AAA	GTA	TTG	CGA	GAA	TTG	ATG	GTA	AAA	GCC	ACG	AAG	ACT	CGT	1660
	Lys	Lys	Lys	Val	Leu	Arg	Glu	Leu	Met	Val	Lys	Ala	Thr	Lys	Thr	Arg	
					435					440					445		
10	GAT	ATC	ACC	TGG	TAC	ATA	GAG	CCA	AGT	GAA	TTT	AAA	TGC	GAG	GAC	AAG	1708
	Asp	Ile	Thr	Trp	Tyr	Ile	Glu	Pro	Ser	Glu	Phe	Lys	Cys	Glu	Asp	Lys	
				450					455					460			
15	GTC	AGG	TTA	TAC	TAT	AAC	AAA	AGT	TCA	GGT	CCT	CTC	TCC	CAT	GCT	AAG	1756
	Val	Arg	Leu	Tyr	Tyr	Asn	Lys	Ser	Ser	Gly	Pro	Leu	Ser	His	Ala	Lys	
			465					470					475				
20	GAC	TTG	TGG	ATC	CAC	GGA	GGA	TAT	AAT	AAT	TGG	AAG	GAT	GGT	TTG	TCT	1804
	Asp	Leu	Trp	Ile	His	Gly	Gly	Tyr	Asn	Asn	Trp	Lys	Asp	Gly	Leu	Ser	
	480					485						490					
25	ATT	GTC	AAA	AAG	CTT	GTT	AAA	TCT	GAG	AGA	ATA	GAT	GGT	GAT	TGG	TGG	1852
	Ile	Val	Lys	Lys	Leu	Val	Lys	Ser	Glu	Arg	Ile	Asp	Gly	Asp	Trp	Trp	
	495					500					505				510		
30	TAT	ACA	GAG	GTT	GTT	ATT	CCT	GAT	CAG	GCA	CTT	TTC	TTG	GAT	TGG	GTT	1900
	Tyr	Thr	Glu	Val	Val	Ile	Pro	Asp	Gln	Ala	Leu	Phe	Leu	Asp	Trp	Val	
					515					520					525		
35	TTT	GCT	GAT	GGT	CCA	CCC	AAG	CAT	GCC	ATT	GCT	TAT	GAT	AAC	AAT	CAC	1948
	Phe	Ala	Asp	Gly	Pro	Pro	Lys	His	Ala	Ile	Ala	Tyr	Asp	Asn	Asn	His	
				530					535					540			
40	CGC	CAA	GAC	TTC	CAT	GCC	ATT	GTC	CCC	AAC	CAC	ATT	CCG	GAG	GAA	TTA	1996
	Arg	Gln	Asp	Phe	His	Ala	Ile	Val	Pro	Asn	His	Ile	Pro	Glu	Glu	Leu	
			545					550					555				
45	TAT	TGG	GTT	GAG	GAA	GAA	CAT	CAG	ATC	TTT	AAG	ACA	CTT	CAG	GAG	GAG	2044
	Tyr	Trp	Val	Glu	Glu	Glu	His	Gln	Ile	Phe	Lys	Thr	Leu	Gln	Glu	Glu	
	560						565					570					
50	AGA	AGG	CTT	AGA	GAA	GCG	GCT	ATG	CGT	GCT	AAG	GTT	GAA	AAA	ACA	GCA	2092
	Arg	Arg	Leu	Arg	Glu	Ala	Ala	Met	Arg	Ala	Lys	Val	Glu	Lys	Thr	Ala	
	575					580					585					590	
55	CTT	CTG	AAA	ACT	GAA	ACA	AAG	GAA	AGA	ACT	ATG	AAA	TCA	TTT	TTA	CTG	2140
	Leu	Leu	Lys	Thr	Glu	Thr	Lys	Glu	Arg	Thr	Met	Lys	Ser	Phe	Leu	Leu	
				595						600					605		
60	TCT	CAG	AAG	CAT	GTA	GTA	TAT	ACT	GAA	CCT	CTT	GAT	ATC	CAA	GCT	GGA	2188
	Ser	Gln	Lys	His	Val	Val	Tyr	Thr	Glu	Pro	Leu	Asp	Ile	Gln	Ala	Gly	
				610					615					620			
65	AGC	AGC	GTC	ACA	GTT	TAC	TAT	AAT	CCC	GCC	AAT	ACA	GTA	CTT	AAT	GGT	2236
	Ser	Ser	Val	Thr	Val	Tyr	Tyr	Asn	Pro	Ala	Asn	Thr	Val	Leu	Asn	Gly	
			625					630					635				

EP 0 779 363 A2

5	AAA CCT GAA ATT TGG TTC AGA TGT TCA TTT AAT CGC TGG ACT CAC CGC 2284 Lys Pro Glu Ile Trp Phe Arg Cys Ser Phe Asn Arg Trp Thr His Arg 640 645 650
10	CTG GGT CCA TTG CCA CCT CAG AAA ATG TCG CCT GCT GAA AAT GGC ACC 2332 Leu Gly Pro Leu Pro Pro Gln Lys Met Ser Pro Ala Glu Asn Gly Thr 655 660 665 670
15	CAT GTC AGA GCA ACT GTG AAG GTT CCA TTG GAT GCA TAT ATG ATG GAT 2380 His Val Arg Ala Thr Val Lys Val Pro Leu Asp Ala Tyr Met Met Asp 675 680 685
20	TTT GTA TTT TCC GAG AGA GAA GAT GGT GGG ATT TTT GAC AAT AAG AGC 2428 Phe Val Phe Ser Glu Arg Glu Asp Gly Gly Ile Phe Asp Asn Lys Ser 690 695 700
25	GGA ATG GAC TAT CAC ATA CCT GTG TTT GGA GGA GTC GCT AAA GAA CCT 2476 Gly Met Asp Tyr His Ile Pro Val Phe Gly Gly Val Ala Lys Glu Pro 705 710 715
30	CCA ATG CAT ATT GTC CAT ATT GCT GTC GAA ATG GCA CCA ATT GCA AAG 2524 Pro Met His Ile Val His Ile Ala Val Glu Met Ala Pro Ile Ala Lys 720 725 730
35	GTG GGA GGC CTT GGT GAT GTT GTT ACT AGT CTT TCC CGT GCT GTT CAA 2572 Val Gly Gly Leu Gly Asp Val Val Thr Ser Leu Ser Arg Ala Val Gln 735 740 745 750
40	GAT TTA AAC CAT AAT GTG GAT ATT ATC TTA CCT AAG TAT GAC TGT TTG 2620 Asp Leu Asn His Asn Val Asp Ile Ile Leu Pro Lys Tyr Asp Cys Leu 755 760 765
45	AAG ATG AAT AAT GTG AAG GAC TTT CGG TTT CAC AAA AAC TAC TTT TGG 2668 Lys Met Asn Asn Val Lys Asp Phe Arg Phe His Lys Asn Tyr Phe Trp 770 775 780
50	GGT GGG ACT GAA ATA AAA GTA TGG TTT GGA AAG GTG GAA GGT CTC TCG 2716 Gly Gly Thr Glu Ile Lys Val Trp Phe Gly Lys Val Glu Gly Leu Ser 785 790 795
55	GTC TAT TTT TTG GAG CCT CAA AAC GGG TTA TTT TCG AAA GGG TGC GTC 2764 Val Tyr Phe Leu Glu Pro Gln Asn Gly Leu Phe Ser Lys Gly Cys Val 800 805 810
60	TAT GGT TGT AGC AAC GAT GGT GAA CGA TTT GGT TTC TTC TGT CAC GCG 2812 Tyr Gly Cys Ser Asn Asp Gly Glu Arg Phe Gly Phe Phe Cys His Ala 815 820 825 830
65	GCT TTG GAG TTT CTT CTG CAA GGT GGA TTT AGT CCG GAT ATC ATT CAT 2860 Ala Leu Glu Phe Leu Leu Gln Gly Gly Phe Ser Pro Asp Ile Ile His 835 840 845
70	TGC CAT GAT TGG TCT AGT GCT CCT GTT GCT TGG CTC TTT AAG GAA CAA 2908 Cys His Asp Trp Ser Ser Ala Pro Val Ala Trp Leu Phe Lys Glu Gln 850 855 860

EP 0 779 363 A2

	TAT	ACA	CAC	TAT	GGT	CTA	AGC	AAA	TCT	CGT	ATA	GTC	TTC	ACG	ATA	CAT	2956
	Tyr	Thr	His	Tyr	Gly	Leu	Ser	Lys	Ser	Arg	Ile	Val	Phe	Thr	Ile	His	
			865					870					875				
5	AAT	CTT	GAA	TTT	GGG	GCA	GAT	CTC	ATT	GGG	AGA	GCA	ATG	ACT	AAC	GCA	3004
	Asn	Leu	Glu	Phe	Gly	Ala	Asp	Leu	Ile	Gly	Arg	Ala	Met	Thr	Asn	Ala	
		880					885					890					
10	GAC	AAA	GCT	ACA	ACA	GTT	TCA	CCA	ACT	TAC	TCA	CAG	GAG	GTG	TCT	GGA	3052
	Asp	Lys	Ala	Thr	Thr	Val	Ser	Pro	Thr	Tyr	Ser	Gln	Glu	Val	Ser	Gly	
		895				900					905					910	
15	AAC	CCT	GTA	ATT	GCG	CCT	CAC	CTT	CAC	AAG	TTC	CAT	GGT	ATA	GTG	AAT	3100
	Asn	Pro	Val	Ile	Ala	Pro	His	Leu	His	Lys	Phe	His	Gly	Ile	Val	Asn	
					915					920					925		
20	GGG	ATT	GAC	CCA	GAT	ATT	TGG	GAT	CCT	TTA	AAC	GAT	AAG	TTC	ATT	CCG	3148
	Gly	Ile	Asp	Pro	Asp	Ile	Trp	Asp	Pro	Leu	Asn	Asp	Lys	Phe	Ile	Pro	
				930					935						940		
25	ATT	CCG	TAC	ACT	TCA	GAA	AAC	GTT	GTT	GAG	GGC	AAA	ACA	GCA	GCC	AAG	3196
	Ile	Pro	Tyr	Thr	Ser	Glu	Asn	Val	Val	Glu	Gly	Lys	Thr	Ala	Ala	Lys	
			945					950					955				
30	GAA	GCT	TTG	CAG	CGA	AAA	CTT	GGA	CTG	AAA	CAG	GCT	GAC	CTT	CCT	TTG	3244
	Glu	Ala	Leu	Gln	Arg	Lys	Leu	Gly	Leu	Lys	Gln	Ala	Asp	Leu	Pro	Leu	
		960					965					970					
35	GTA	GGA	ATT	ATC	ACC	CGC	TTA	ACT	CAC	CAG	AAA	GGA	ATC	CAC	CTC	ATT	3292
	Val	Gly	Ile	Ile	Thr	Arg	Leu	Thr	His	Gln	Lys	Gly	Ile	His	Leu	Ile	
		975				980					985					990	
40	AAA	CAT	GCT	ATT	TGG	CGC	ACC	TTG	GAA	CGG	AAC	GGA	CAG	GTA	GTC	TTG	3340
	Lys	His	Ala	Ile	Trp	Arg	Thr	Leu	Glu	Arg	Asn	Gly	Gln	Val	Val	Leu	
					995					1000					1005		
45	CTT	GGT	TCT	GCT	CCT	GAT	CCT	AGG	GTA	CAA	AAC	AAT	TTT	GTT	AAT	TTG	3388
	Leu	Gly	Ser	Ala	Pro	Asp	Pro	Arg	Val	Gln	Asn	Asn	Phe	Val	Asn	Leu	
				1010					1015					1020			
50	GCA	AAT	CAA	TTG	CAC	TCC	AAA	TAT	AAT	GAC	CGC	GCA	CGA	CTC	TGT	CTA	3436
	Ala	Asn	Gln	Leu	His	Ser	Lys	Tyr	Asn	Asp	Arg	Ala	Arg	Leu	Cys	Leu	
			1025					1030					1035				
55	ACA	TAT	GAC	GAG	CCA	CTT	TCT	CAC	CTG	ATA	TAT	GCT	GGT	GCT	GAT	TTT	3484
	Thr	Tyr	Asp	Glu	Pro	Leu	Ser	His	Leu	Ile	Tyr	Ala	Gly	Ala	Asp	Phe	
			1040				1045					1050					
60	ATT	CTA	GTT	CCT	TCA	ATA	TTT	GAG	CCA	TGT	GGA	CTA	ACA	CAA	CTT	ACC	3532
	Ile	Leu	Val	Pro	Ser	Ile	Phe	Glu	Pro	Cys	Gly	Leu	Thr	Gln	Leu	Thr	
		1055				1060					1065					1070	
65	GCT	ATG	AGA	TAT	GGT	TCA	ATT	CCA	GTC	GTG	CGT	AAA	ACT	GGA	GGA	CTT	3580
	Ala	Met	Arg	Tyr	Gly	Ser	Ile	Pro	Val	Val	Arg	Lys	Thr	Gly	Gly	Leu	
					1075					1080					1085		

TAT GAT ACT GTA TTT GAT GTT GAC CAT GAC AAA GAG AGA GCA CAA CAG 3628  
 Tyr Asp Thr Val Phe Asp Val Asp His Asp Lys Glu Arg Ala Gln Gln  
 1090 1095 1100

TGT GGT CTT GAA CCA AAT GGA TTC AGC TTT GAT GGA GCA GAT GCT GGC 3676  
 Cys Gly Leu Glu Pro Asn Gly Phe Ser Phe Asp Gly Ala Asp Ala Gly  
 1105 1110 1115

GGA GTT GAT TAT GCT CTG AAT AGA GCT CTC TCT GCT TGG TAC GAT GGT 3724  
 Gly Val Asp Tyr Ala Leu Asn Arg Ala Leu Ser Ala Trp Tyr Asp Gly  
 1120 1125 1130

CGG GAT TGG TTC AAC TCT TTA TGC AAG CAG GTC ATG GAA CAA GAT TGG 3772  
 Arg Asp Trp Phe Asn Ser Leu Cys Lys Gln Val Met Glu Gln Asp Trp  
 1135 1140 1145 1150

TCT TGG AAC CGA CCT GCT CTT GAT TAT TTG GAG CTT TAC CAT GCT GCT 3820  
 Ser Trp Asn Arg Pro Ala Leu Asp Tyr Leu Glu Leu Tyr His Ala Ala  
 1155 1160 1165

AGA AAG TTA GAA TAG TTAGTTTGTG AGATGCTAGC AGAAAAATTC ACGAGATCTG 3875  
 Arg Lys Leu Glu \*  
 1170

CAATCTGTAC AGGTTTCAGTG TTTGGGTCTG GACAGCTTTA TCATTTTCCTA TATCAAAGTA 3935

TAAATCAAGT CTACACTGAG GATCAATAGC AGACAGTCCT CAAGTTCATT TCATTTTTTTG 3995

GGGCAAAACAT ATGAAAGAGC TTAGCCTCTT AATAATGTCG GCCTATTGAT GATTATTTTGT 4055

TTTGGGAAGA AATGAGAAAT CAAAGGATGC AAAATAAAAA AAAAAAAAAA AAAAAAACT 4115

CGTGCCGAAT TC 4127

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1231 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Met Asp Val Pro Phe Pro Leu His Arg Ser Leu Ser Cys Thr Ser Val  
 -60 -55 -50 -45

Ser Asn Ala Ile Thr His Leu Lys Ile Lys Pro Ile Leu Gly Phe Val  
 -40 -35 -30

Ser His Gly Thr Thr Ser Leu Ser Val Gln Ser Ser Ser Trp Arg Lys  
 -25 -20 -15

Asp Gly Met Val Thr Gly Val Ser Phe Ser Ile Cys Ala Asn Phe Ser  
 -10 -5 1



EP 0 779 363 A2

Gly Arg Arg Arg Arg Lys Val Ser Thr Pro Arg Ser Gln Gly Ser Ser  
 5 10 15 20  
 5 Pro Lys Gly Phe Val Pro Arg Lys Pro Ser Gly Met Ser Thr Gln Arg  
 25 30 35  
 Lys Val Gln Lys Ser Asn Gly Asp Lys Glu Ser Lys Ser Thr Ser Thr  
 40 45 50  
 10 Ser Lys Glu Ser Glu Ile Ser Asn Gln Lys Thr Val Glu Ala Arg Val  
 55 60 65  
 Glu Thr Ser Asp Asp Asp Thr Lys Gly Val Val Arg Asp His Lys Phe  
 70 75 80  
 15 Leu Glu Asp Glu Asp Glu Ile Asn Gly Ser Thr Lys Ser Ile Ser Met  
 85 90 95 100  
 20 Ser Pro Val Arg Val Ser Ser Gln Phe Val Glu Ser Glu Glu Thr Gly  
 105 110 115  
 Gly Asp Asp Lys Asp Ala Val Lys Leu Asn Lys Ser Lys Arg Ser Glu  
 120 125 130  
 25 Glu Ser Gly Phe Ile Ile Asp Ser Val Ile Arg Glu Gln Ser Gly Ser  
 135 140 145  
 Gln Gly Glu Thr Asn Ala Ser Ser Lys Gly Ser His Ala Val Gly Thr  
 150 155 160  
 30 Lys Leu Tyr Glu Ile Leu Gln Val Asp Val Glu Pro Gln Gln Leu Lys  
 165 170 175 180  
 35 Glu Asn Asn Ala Gly Asn Val Glu Tyr Lys Gly Pro Val Ala Ser Lys  
 185 190 195  
 Leu Leu Glu Ile Thr Lys Ala Ser Asp Val Glu His Thr Glu Ser Asn  
 200 205 210  
 40 Glu Ile Asp Asp Leu Asp Thr Asn Ser Phe Phe Lys Ser Asp Leu Ile  
 215 220 225  
 Glu Glu Asp Glu Pro Leu Ala Ala Gly Thr Val Glu Thr Gly Asp Ser  
 230 235 240  
 45 Ser Leu Asn Leu Arg Leu Glu Met Glu Ala Asn Leu Arg Arg Gln Ala  
 245 250 255 260  
 50 Ile Glu Arg Leu Ala Glu Glu Asn Leu Leu Gln Gly Ile Arg Leu Phe  
 265 270 275  
 Cys Phe Pro Glu Val Val Lys Pro Asp Glu Asp Val Glu Ile Phe Leu  
 280 285 290  
 55 Asn Arg Gly Leu Ser Thr Leu Lys Asn Glu Ser Asp Val Leu Ile Met  
 295 300 305

EP 0 779 363 A2

	Gly	Ala	Phe	Asn	Glu	Trp	Arg	Tyr	Arg	Ser	Phe	Thr	Thr	Arg	Leu	Thr
		310					315					320				
5	Glu	Thr	His	Leu	Asn	Gly	Asp	Trp	Trp	Ser	Cys	Lys	Ile	His	Val	Pro
	325					330					335					340
	Lys	Glu	Ala	Tyr	Arg	Ala	Asp	Phe	Val	Phe	Phe	Asn	Gly	Gln	Asp	Val
					345					350					355	
10	Tyr	Asp	Asn	Asn	Asp	Gly	Asn	Asp	Phe	Ser	Ile	Thr	Val	Lys	Gly	Gly
			360						365					370		
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15			375					380					385			
	Glu	Gln	Glu	Lys	Leu	Ala	Lys	Glu	Gln	Ala	Glu	Arg	Glu	Arg	Leu	Ala
		390					395					400				
20	Glu	Glu	Gln	Arg	Arg	Ile	Glu	Ala	Glu	Lys	Ala	Glu	Ile	Glu	Ala	Asp
	405					410					415					420
	Arg	Ala	Gln	Ala	Lys	Glu	Glu	Ala	Ala	Lys	Lys	Lys	Lys	Val	Leu	Arg
					425					430					435	
25	Glu	Leu	Met	Val	Lys	Ala	Thr	Lys	Thr	Arg	Asp	Ile	Thr	Trp	Tyr	Ile
			440						445					450		
	Glu	Pro	Ser	Glu	Phe	Lys	Cys	Glu	Asp	Lys	Val	Arg	Leu	Tyr	Tyr	Asn
30			455					460				465				
	Lys	Ser	Ser	Gly	Pro	Leu	Ser	His	Ala	Lys	Asp	Leu	Trp	Ile	His	Gly
		470					475					480				
35	Gly	Tyr	Asn	Asn	Trp	Lys	Asp	Gly	Leu	Ser	Ile	Val	Lys	Lys	Leu	Val
	485					490					495					500
	Lys	Ser	Glu	Arg	Ile	Asp	Gly	Asp	Trp	Trp	Tyr	Thr	Glu	Val	Val	Ile
					505				510						515	
40	Pro	Asp	Gln	Ala	Leu	Phe	Leu	Asp	Trp	Val	Phe	Ala	Asp	Gly	Pro	Pro
			520						525					530		
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45			535					540					545			
	Ile	Val	Pro	Asn	His	Ile	Pro	Glu	Glu	Leu	Tyr	Trp	Val	Glu	Glu	Glu
		550					555					560				
50	His	Gln	Ile	Phe	Lys	Thr	Leu	Gln	Glu	Glu	Arg	Arg	Leu	Arg	Glu	Ala
	565					570					575					580
	Ala	Met	Arg	Ala	Lys	Val	Glu	Lys	Thr	Ala	Leu	Leu	Lys	Thr	Glu	Thr
					585					590					595	
55	Lys	Glu	Arg	Thr	Met	Lys	Ser	Phe	Leu	Leu	Ser	Gln	Lys	His	Val	Val
				600					605					610		

EP 0 779 363 A2

	Tyr	Thr	Glu	Pro	Leu	Asp	Ile	Gln	Ala	Gly	Ser	Ser	Val	Thr	Val	Tyr
			615					620					625			
5	Tyr	Asn	Pro	Ala	Asn	Thr	Val	Leu	Asn	Gly	Lys	Pro	Glu	Ile	Trp	Phe
		630					635					640				
	Arg	Cys	Ser	Phe	Asn	Arg	Trp	Thr	His	Arg	Leu	Gly	Pro	Leu	Pro	Pro
	645					650					655					660
10	Gln	Lys	Met	Ser	Pro	Ala	Glu	Asn	Gly	Thr	His	Val	Arg	Ala	Thr	Val
					665					670					675	
	Lys	Val	Pro	Leu	Asp	Ala	Tyr	Met	Met	Asp	Phe	Val	Phe	Ser	Glu	Arg
15				680					685					690		
	Glu	Asp	Gly	Gly	Ile	Phe	Asp	Asn	Lys	Ser	Gly	Met	Asp	Tyr	His	Ile
			695					700					705			
20	Pro	Val	Phe	Gly	Gly	Val	Ala	Lys	Glu	Pro	Pro	Met	His	Ile	Val	His
		710					715					720				
	Ile	Ala	Val	Glu	Met	Ala	Pro	Ile	Ala	Lys	Val	Gly	Gly	Leu	Gly	Asp
	725					730					735					740
25	Val	Val	Thr	Ser	Leu	Ser	Arg	Ala	Val	Gln	Asp	Leu	Asn	His	Asn	Val
					745					750					755	
	Asp	Ile	Ile	Leu	Pro	Lys	Tyr	Asp	Cys	Leu	Lys	Met	Asn	Asn	Val	Lys
30				760					765					770		
	Asp	Phe	Arg	Phe	His	Lys	Asn	Tyr	Phe	Trp	Gly	Gly	Thr	Glu	Ile	Lys
			775					780					785			
35	Val	Trp	Phe	Gly	Lys	Val	Glu	Gly	Leu	Ser	Val	Tyr	Phe	Leu	Glu	Pro
		790					795					800				
	Gln	Asn	Gly	Leu	Phe	Ser	Lys	Gly	Cys	Val	Tyr	Gly	Cys	Ser	Asn	Asp
	805					810					815					820
40	Gly	Glu	Arg	Phe	Gly	Phe	Phe	Cys	His	Ala	Ala	Leu	Glu	Phe	Leu	Leu
					825					830					835	
	Gln	Gly	Gly	Phe	Ser	Pro	Asp	Ile	Ile	His	Cys	His	Asp	Trp	Ser	Ser
				840					845					850		
45	Ala	Pro	Val	Ala	Trp	Leu	Phe	Lys	Glu	Gln	Tyr	Thr	His	Tyr	Gly	Leu
			855					860					865			
50	Ser	Lys	Ser	Arg	Ile	Val	Phe	Thr	Ile	His	Asn	Leu	Glu	Phe	Gly	Ala
		870					875					880				
	Asp	Leu	Ile	Gly	Arg	Ala	Met	Thr	Asn	Ala	Asp	Lys	Ala	Thr	Thr	Val
	885					890					895					900
55	Ser	Pro	Thr	Tyr	Ser	Gln	Glu	Val	Ser	Gly	Asn	Pro	Val	Ile	Ala	Pro
					905					910					915	

His Leu His Lys Phe His Gly Ile Val Asn Gly Ile Asp Pro Asp Ile  
 920 925 930  
 5 Trp Asp Pro Leu Asn Asp Lys Phe Ile Pro Ile Pro Tyr Thr Ser Glu  
 935 940 945  
 Asn Val Val Glu Gly Lys Thr Ala Ala Lys Glu Ala Leu Gln Arg Lys  
 950 955 960  
 10 Leu Gly Leu Lys Gln Ala Asp Leu Pro Leu Val Gly Ile Ile Thr Arg  
 965 970 975 980  
 15 Leu Thr His Gln Lys Gly Ile His Leu Ile Lys His Ala Ile Trp Arg  
 985 990 995  
 Thr Leu Glu Arg Asn Gly Gln Val Val Leu Leu Gly Ser Ala Pro Asp  
 1000 1005 1010  
 20 Pro Arg Val Gln Asn Asn Phe Val Asn Leu Ala Asn Gln Leu His Ser  
 1015 1020 1025  
 25 Lys Tyr Asn Asp Arg Ala Arg Leu Cys Leu Thr Tyr Asp Glu Pro Leu  
 1030 1035 1040  
 Ser His Leu Ile Tyr Ala Gly Ala Asp Phe Ile Leu Val Pro Ser Ile  
 1045 1050 1055 1060  
 30 Phe Glu Pro Cys Gly Leu Thr Gln Leu Thr Ala Met Arg Tyr Gly Ser  
 1065 1070 1075  
 Ile Pro Val Val Arg Lys Thr Gly Gly Leu Tyr Asp Thr Val Phe Asp  
 1080 1085 1090  
 35 Val Asp His Asp Lys Glu Arg Ala Gln Gln Cys Gly Leu Glu Pro Asn  
 1095 1100 1105  
 40 Gly Phe Ser Phe Asp Gly Ala Asp Ala Gly Gly Val Asp Tyr Ala Leu  
 1110 1115 1120  
 Asn Arg Ala Leu Ser Ala Trp Tyr Asp Gly Arg Asp Trp Phe Asn Ser  
 1125 1130 1135 1140  
 45 Leu Cys Lys Gln Val Met Glu Gln Asp Trp Ser Trp Asn Arg Pro Ala  
 1145 1150 1155  
 50 Leu Asp Tyr Leu Glu Leu Tyr His Ala Ala Arg Lys Leu Glu \*  
 1160 1165 1170

## 55 Claims

1. Altered starch extracted from a transformed potato plant or the progeny thereof, which starch, as extracted, has a viscosity onset temperature as determined by differential scanning calorimetry which is reduced by at least 5°C

compared to starch extracted from equivalent, non-transformed plants.

2. Altered starch according to claim 1, wherein the viscosity onset temperature is reduced by at least 7°C compared to starch extracted from equivalent, non-transformed plants.
3. Altered starch extracted from a transformed potato plant or the progeny thereof, which starch, as extracted, has a viscosity onset temperature as determined by differential scanning calorimetry, of less than 60°C.
4. Altered starch according to claim 3 which, as extracted, has a viscosity onset temperature of less than 55°C.
5. Altered starch according to any one of claims 1-4 which, as extracted, has a reduced endotherm peak temperature (as extracted) as determined by differential scanning calorimetry compared to starch extracted from equivalent, non-transformed plants.
6. Altered starch according to any one of claims 1-5 which, as extracted, has an endotherm peak temperature as determined by differential scanning calorimetry which is reduced by at least 5°C compared to starch extracted from equivalent, non-transformed plants.
7. Altered starch according to any one of claims 1-6 which, as extracted, has an endotherm peak temperature as determined by differential scanning calorimetry, of less than 59°C.
8. Altered starch according to any one of the preceding claims, having a substantially normal amylose content.
9. A polypeptide obtainable from a soluble extract of potato tubers and having starch synthase activity, in substantially isolated form.
10. A polypeptide according to claim 9, having an apparent molecular weight, as judged by SDS-PAGE, in the range of 100-140 kDa, or a functional equivalent thereof.
11. A polypeptide according to claim 9 or 10, having an apparent molecular weight, as judged by SDS-PAGE, of 140 kDa.
12. A polypeptide according to claim 9 or 10, having an apparent molecular weight, as judged by SDS-PAGE, of 120 kDa.
13. A polypeptide according to claim 9 or 10, having an apparent molecular weight, as judged by SDS-PAGE, of 110 kDa.
14. A polypeptide according to claim 9 or 10, obtainable from developing tubers of *S. tuberosum* cultivar Désirée, having an apparent molecular weight, as judged by SDS-PAGE, of 100 kDa.
15. A polypeptide according to any one of claims 9-14, comprising the amino acid sequence shown in Figure 6.
16. A nucleic acid sequence comprising at least 200bp and exhibiting at least 80% sequence identity with the corresponding region of the DNA sequence shown in Figure 6, operably linked in the sense or antisense orientation to a promoter operable in a plant.
17. A sequence according to claim 16 comprising at least 300-600bp.
18. A sequence according to claim 16 or 17, exhibiting at least 85% sequence identity with the corresponding region of the DNA sequence shown in Figure 6.
19. A sequence according to any one of claims 16, 17 or 18 exhibiting at least 90% sequence identity with the corresponding region of the DNA sequence shown in Figure 6.
20. A sequence according to any one of claims 16-19, comprising a 5' and/or a 3' untranslated region.
21. A sequence according to any one of claims 16-20, encoding at least a portion of a polypeptide in accordance with

any one of claims 9-15.

22. A sequence according to any one of claims 16-21, excluding sequences disclosed in WO 96/15248.

5 23. A nucleic acid construct comprising the nucleic acid sequence of any one of claims 16-22.

24. A host cell into which has been introduced a nucleic acid sequence according to any one of claims 16-22.

10 25. A host cell according to claim 24, wherein the nucleic acid sequence is introduced in a construct according to claim 23.

26. A host cell according to claim 24 or 25, wherein the introduced sequence is integrated into the host cell genome.

15 27. A plant host cell according to any one of claims 24, 25 or 26.

28. A plant or part thereof, into which has been introduced a nucleic acid sequence according to any one of claims 16-22, or the progeny of such a plant or part thereof.

20 29. A plant or part thereof according to claim 28, wherein the plant is selected from the group consisting of : potato, tomato, rice, wheat, peal cassava, sweet potato, barley, oat and maize.

30. A plant according to claim 28 or 29, comprising starch in accordance with any one of claims 1-8.

25 31. Starch extracted from a plant according to claim 28 or 29.

32. Starch according to claim 31, having altered properties, as extracted, relative to starch extracted from equivalent but untransformed plants.

30 33. Starch according to claim 31 or 32, and in accordance with any one of claims 1-8.

35 34. A method of producing altered starch from transformed potato plants or their progeny, the method comprising extracting starch from a potato plant, at least the tubers of which comprise a nucleic acid sequence in accordance with any one of claims 16-22, said sequence having been artificially introduced into the potato plant or a predecessor thereof.

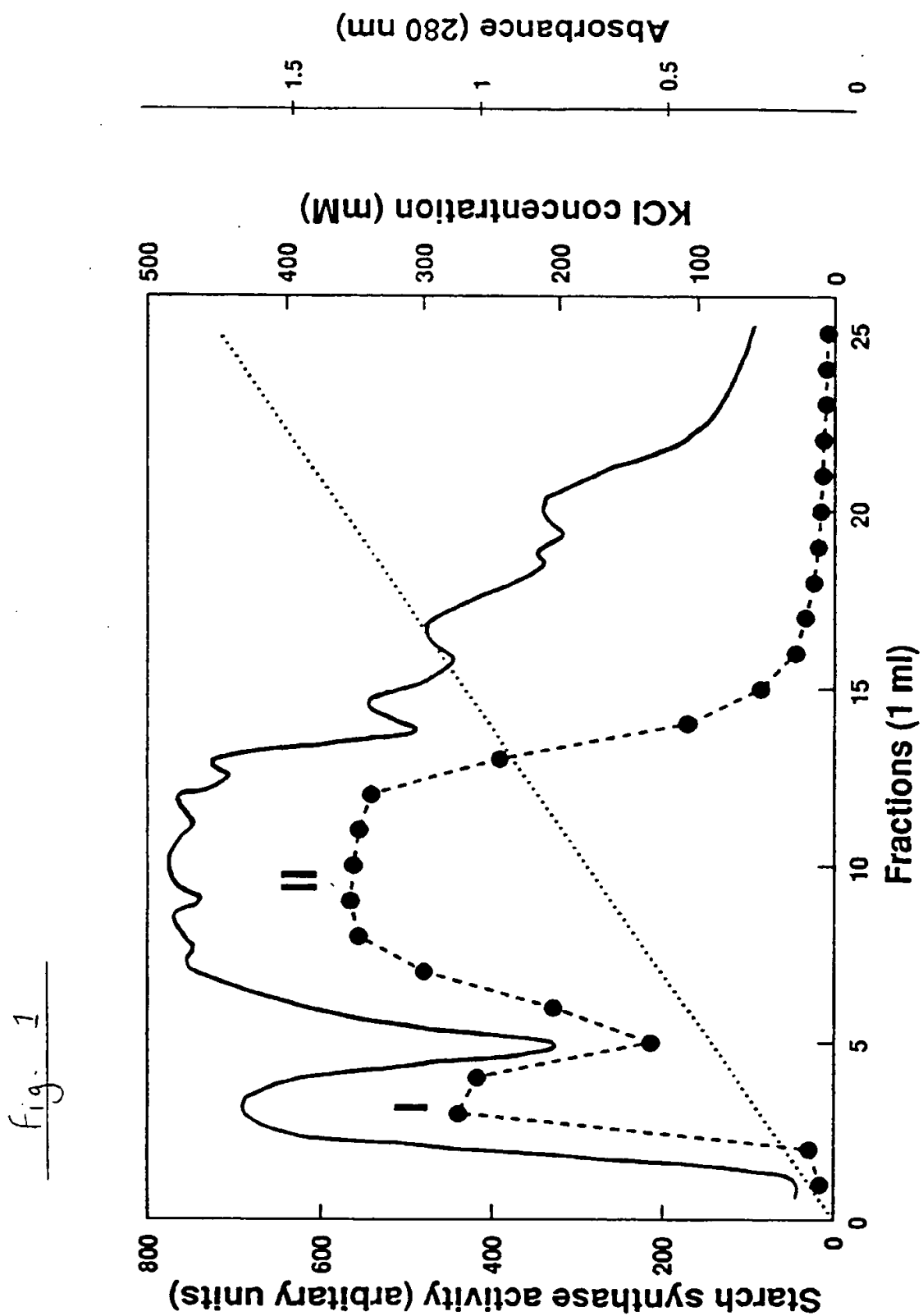


Fig. 2

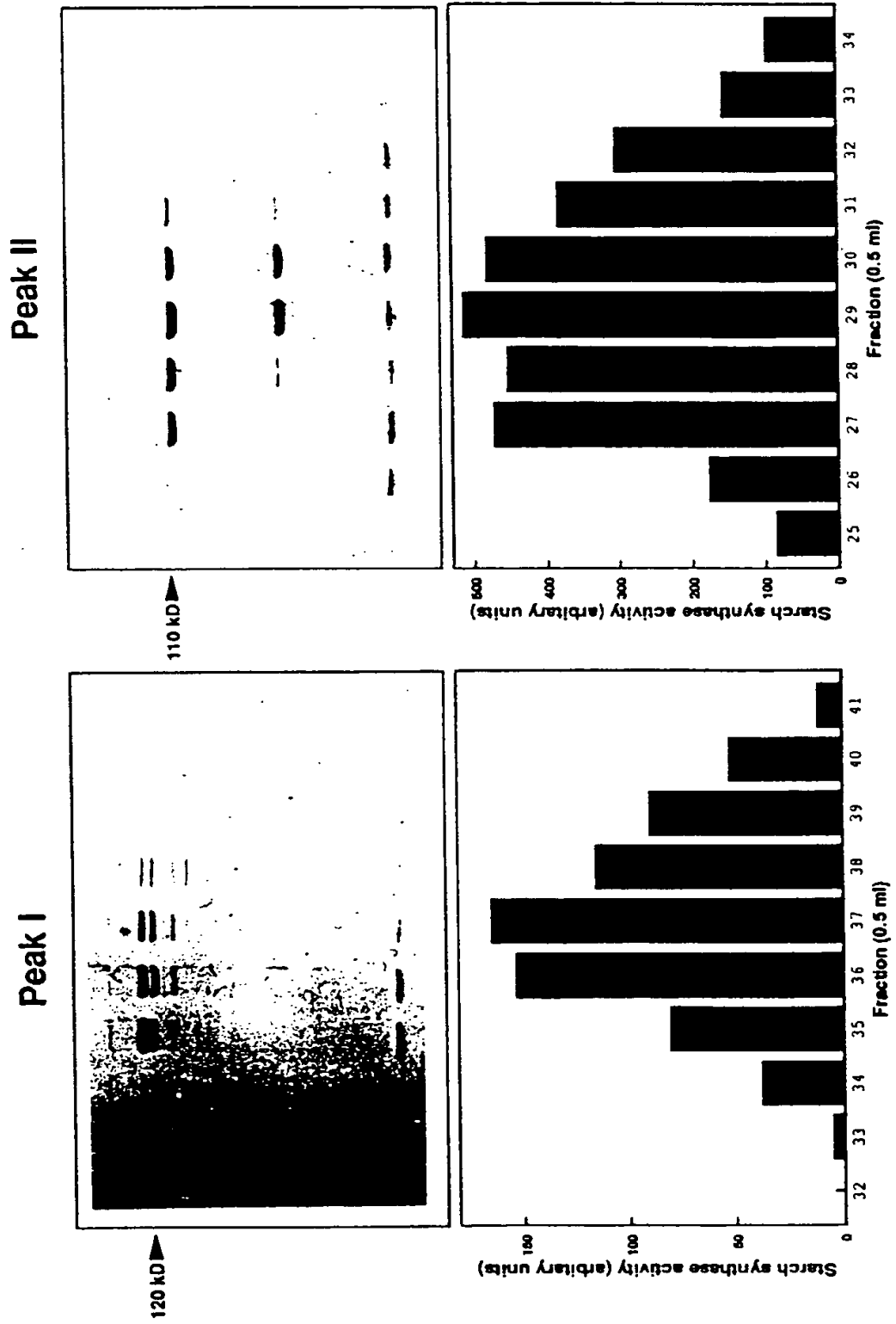




Fig. 3

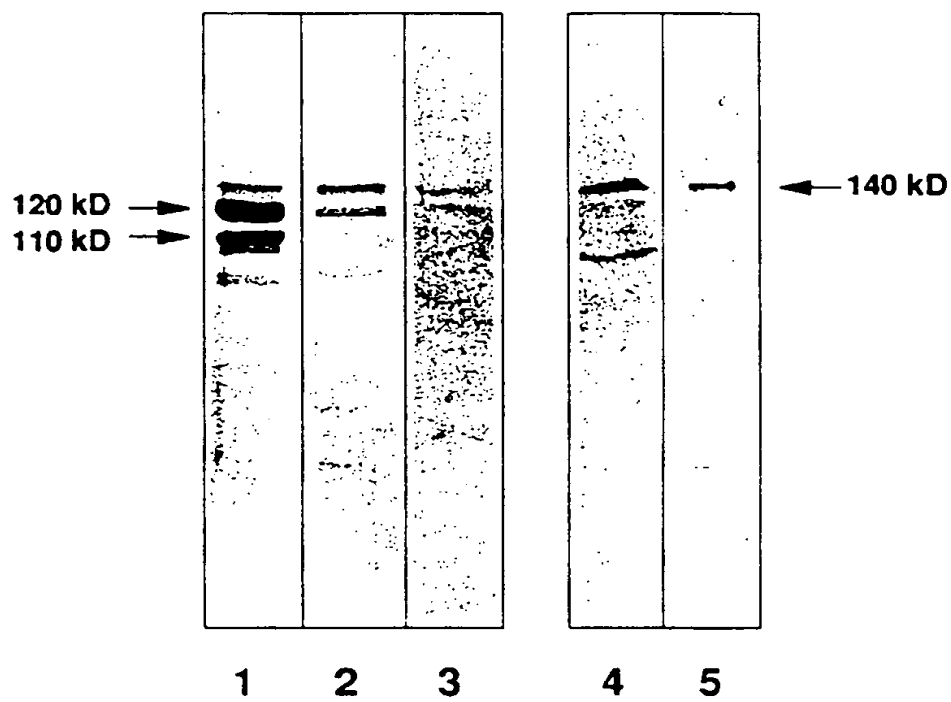


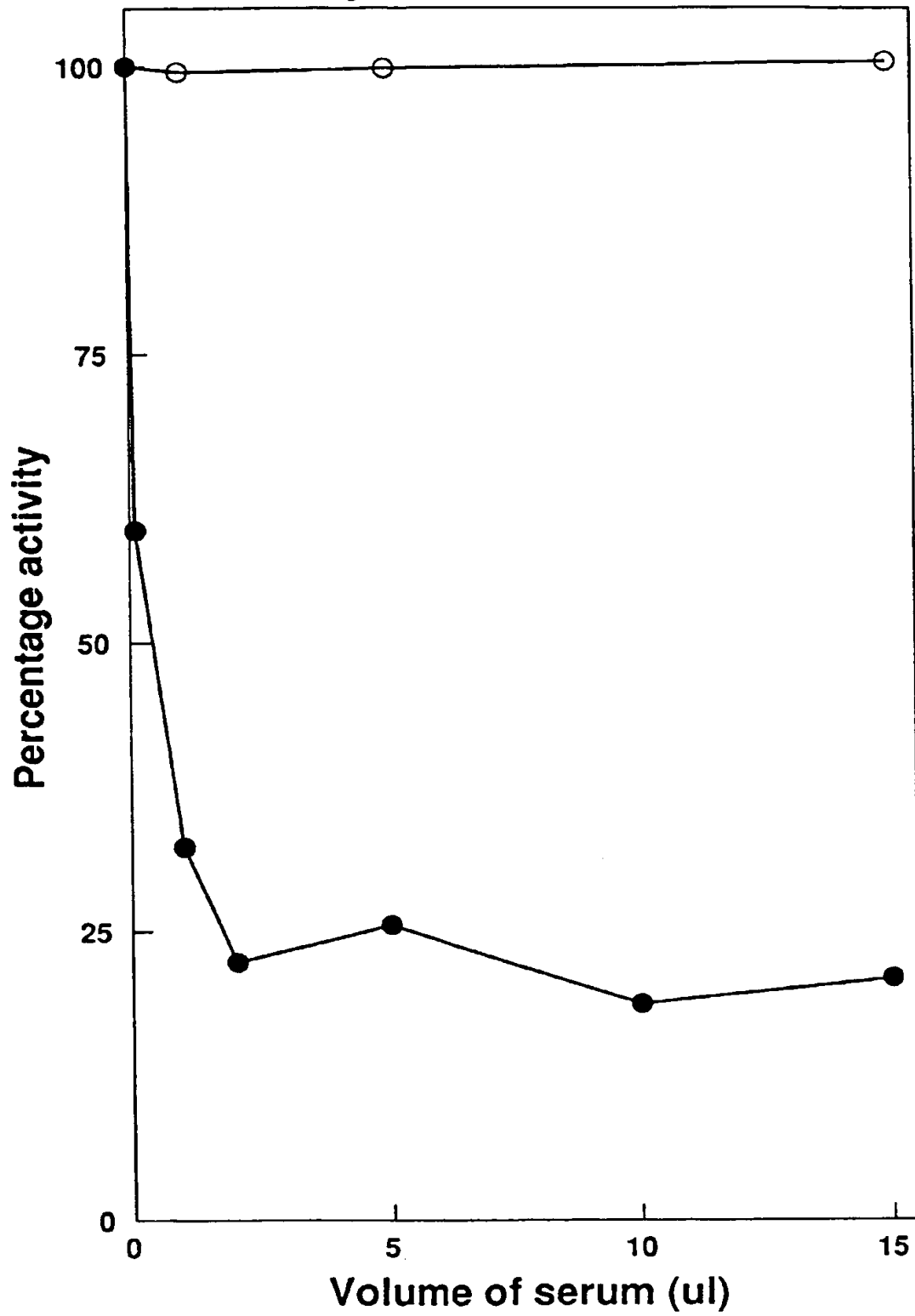
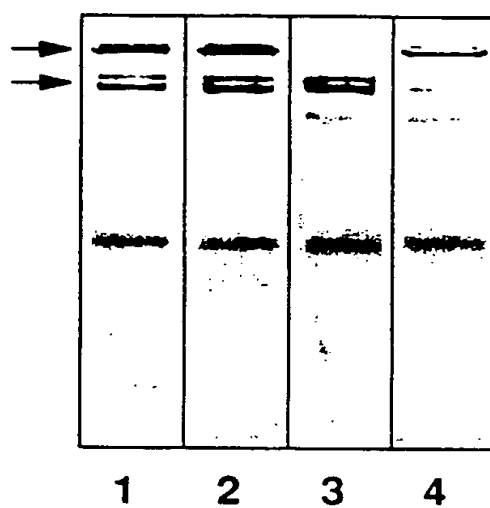
Fig. 4

Fig. 5



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TGGTGTCTGTGGGTACCGATGGCATGTGGCAATTGCCACTGCCATAGATCATTGAGTTGCCACAAGTGCTCCCAATTCATAACCCACCTCAAGATCAAACTATTCTTGGGTGTGCTCTC  
 AACCAAGAACACACCCCACTGGTACCCTACAAGGTAAAGGTGAGCTATCTAGTAACTCAACGTGTTCCACAGAGGTTACGCTATTCTGGGCGAGGTTCTAGTTTGGATAAGAACCTCAACAGAGG

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TGTATGGAAACACAAAGTCATCAGTACAATCTCTTCATGAGGAGGAGGATGGATGGTACTCGGGGTTCTATTTCATTCTTGCAAAATCTCTGGGAAGAAGACGGAGAAAAGTTTCAAC  
 AGTACTTCTGCTGTCACATAGTCATGTCAGAGAGAGTACTCTCTCTACCTTACCAATGACCCCAAGTAAAAGGTAAACACGTTTAAAGAGCCCTCTCTCTTCTGCGCTCTTTTCAAAAGTTG

H S T T S L S V O S S S W R K G G H V T G V S F S I C A Y F S G R R R R K V S T

Scale 1

TCCTAGGAGTCAAGGCTCTTCACTTAAGGGCTTGTGCCAAGGAAGCCCTCAGGATGAGCAGCCAAAGCAAGGTTCAAGAGCAATCGTCATAAAGAAAGTAAAGTACTTCAACATC  
AGGATCTCTAGTCCGAGAAGTGGATTCCCAAGCACTGGTCTCTGGGAAGTCCCTACTCTGTCGGTTCTCTCCAAAGTCTTCTCTGCTACCACTATTCTTTCATTCTCATGAAGTGTGTAG

P R S O G S S P K G F Y P R K P S G M S \* Q R K V Q X S N G O K E S K S I S I S

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K F S E I S N O K T Y E A R Y E I S O O O T X G V V R D H V F L E O E D F I N G

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U T K B I S H S P T R V S S O F V E S E E T G G D D K D A V K L N K S K R S E E

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S C F I I D S V I R E O S G S O G E T N A S S K G S H A , G - K L T E I L G V O

960

Y E P D D : K E N N A G N J E Y K G P V A S K L L E I T K A S D V E H T E S N E

Ps14

GATGATGACTTAGACACATAAGTTCCTTAAATCAGATTTAATTGAAGAGGATGAGCCATTAGCTGACGGAACAGTGAGACTCGAGATTCTCTCTAAACTTAAGATTGAGATGGA  
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: O D L O I N S F F K S D L I E E D E P L A A G T V E T S O S S L N L R L E M E

SnaB I

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TCTTTAGATGCATCTGTCGAGATATCTTTCCGAAACGGCTCTCTTAAATTAACGTCTCCCTAGTCTAATAAAACAAAAGGCTCTCCAACTCTTTGGACTACTCTTACAGCTCTATAAGAAAT

A N L R R D A I E R L A E E N L L O G I R L F C F P E V V K P D E D V E I F L N

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P O L S T L K N E S O V L I H G A F N E W R Y R S F T T G L T E T H L N S O V W

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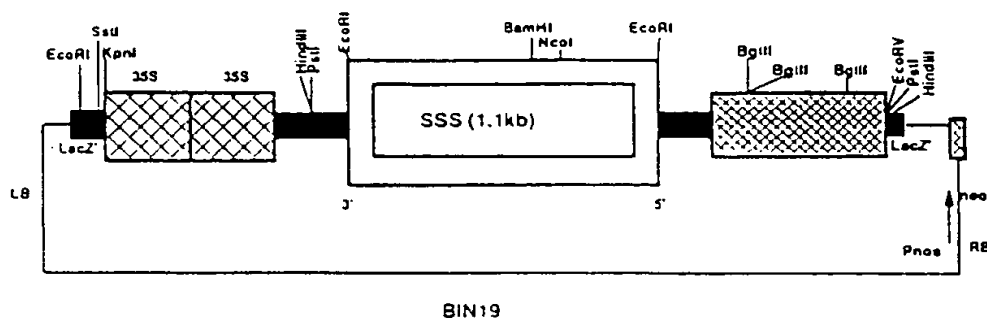
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Fig. 6 (con)

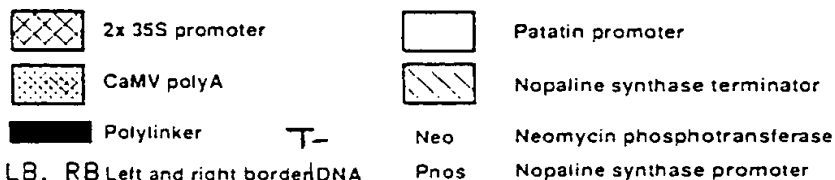
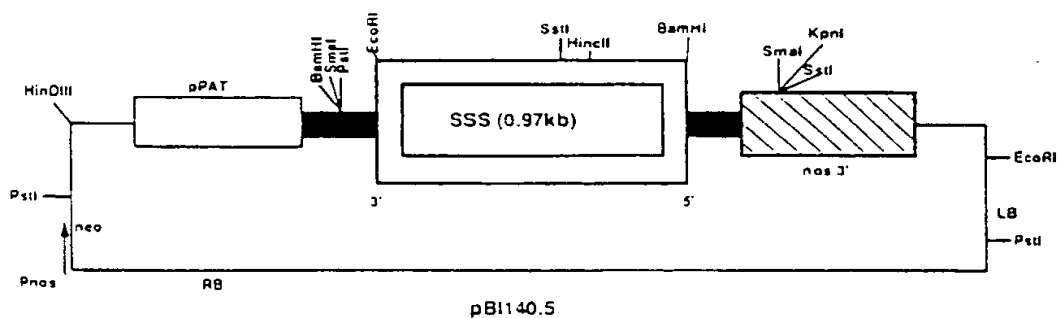
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 Bgl II  
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 P V A W L F K E G I T H I G L S K S R I V F I I H N L E F G A D L I G R A M T N  
 Nco I BstX I  
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 BamH I Hind III  
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 L V G I I T R L T H D K G I H L I K H A I W R T L E R N G G V V L L G S A P D P  
 TAGGGTACAAAACAATTTTGTAAATTTGGCAATCAATTCACCTCCAATATAATGACCGCCACGCACTCTGTCTAACATATGACGAGCCACTTTCTCACCTGATATATGCTGGTGTGA 3480  
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 R V G D N N F V N L A N G L H S K Y N D R A R L C L T Y D E P L S H L I T A G A D  
 Ssp I  
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 AAAAAAGATCAAGGAAGTTATAAATCCGTACACCTGATTTGTGTAATGGGAGATCTATACCAAGTTAAGGTCAGCAGGCATTTTGACCTCTGAAATACTATGACATAACATACA  
 F I L V P S I F E P C U L T O L T A N R Y G S I P V V R K T C G L Y D T V F D V  
 Hind II Sac I  
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 ACTGGTACTGTTTCTCTCTGCTGTGTCTACACAGAACTTGGTTTACCTAAGTGGAACTTACCTGCTTACGACCGCTCAACTAATAGGAGACTTATCTGAGAGAGACGGAACGATGT  
 D H G K E R A D D C G L E P N G F S F D G A D A G G V D Y A L N R A L S A W Y D  
 TGGTGGGATTTGGTCAACTCTTTATGCAAGCAGCTGATGGAACAAGATGGTCTTTGGAACCGACCTGCTCTTGATTATTTGGAGCTTTACCATGCTCTAGAAAGTTAGAATAGTTAGT 3840  
 ACCAGCCCTTAACCAAGTTGAGAAATACGTTGCTGCTAGTACCTTGTCTAACCAGAACCTTGGCTGGACGAGAACTAATAAACCTCGAATGGTACGAGATCTTCAATCTTATCAATCA  
 G R D W F N S L C K D V H E D D W S V N R P A L D Y L E L Y H A A R K L E  
 Bgl II  
 TGTGAGATGCTAGCAGAAAAATTCACGAGATCTGCAATCTGTACAGGTTCAGTGTCTGGCTCTGGACACCTTTATCATTTCCCTATATCAAAAGTATAAACTAAGTCTACACTGAGGATCA 3960  
 GACACTCTACGATCTGTTTTTAAGTGTCTAGACGTTAGACATGTCCAAGTCAAAAACAGACCTTGGAAATAGTAAAGGATATAGTTTATATTTAGTTTCAGATGTAACCTTCTAGT  
 ATAGCAGACAGTCTCAAGTTCAATTTCAATTTTTTGGGCAAAACATATGAAAGAGCTTACCTCTTAATAATGTGCGCTATTGATGATTATTTGCTTTGGGAAGAAATGAGAAATCAAAAG 4080  
 TATGCTGTCTCAGGAGTTCAAGTAAGTAAAAAACCCCTTTGTATACCTTCTCGAATCGGAGAATTATTACAGCCGGATAACTACTAATAAACAAAACCCCTTCTTACTCTTATGTTTC  
 EcoR I  
 GATGCAATATAAAAAAAAAAAAAAAAAAACTCGTCCGCAATTC 4127  
 CTACGTTTTATTTTTTTTTTTTTTTTTTTTGGAGCAGCGCTTAAG

**A**  
pRAT4

Fig. 7



**B**  
pPATRAT







(19)



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(11)

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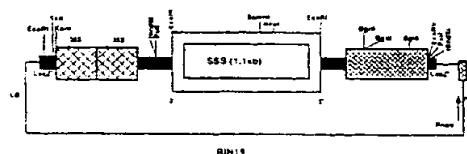
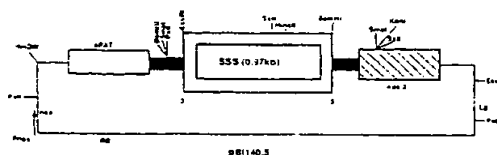
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**(54) Improvements in or relating to soluble starch synthase**

(57) Disclosed is altered starch extracted from a transformed potato plant or the progeny thereof, which starch, as extracted, has a viscosity onset temperature as determined by differential scanning calorimetry which is reduced by at least 5°C compared to starch extracted from equivalent, non-transformed plants, together with a nucleic acid sequence comprising at least 200bp and exhibiting at least 80% sequence identity with the corresponding region of the DNA sequence shown in Figure 6, operably linked in the sense or anti-sense orientation to a promoter operable in a plant, and host cells and plants comprising the sequence.

**A****pRAT4****B****pPATRAT**

- |                                      |                             |
|--------------------------------------|-----------------------------|
| 3.5S promoter                        | Palatin promoter            |
| CamV polyA                           | Hopsite synthase terminator |
| Polylinker                           | Neo                         |
| L.S., R.S. Left and right border DNA | Neomycin phosphotransferase |
|                                      | Hopsite synthase promoter   |



European Patent  
Office

# EUROPEAN SEARCH REPORT

Application Number  
EP 96 30 9004

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
X.D	EDWARDS A ET AL.: "BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF A NOVEL STARCH SYNTHASE FROM POTATO TUBERS" PLANT JOURNAL, vol. 8, no. 2, August 1995, pages 283-294, XP002031375 * the whole document *	9	C12N15/82 C12N15/54 C12N9/10 C08B30/00 C12N5/10 A01H5/00
X	KOSSMANN J. ET AL.: "Transgenic plants as a tool to understand starch biosynthesis" PROGRESS IN BIOTECHNOLOGY, vol. 10, 23 - 26 April 1995, pages 271-278, XP002060102 * see p. 276/77 chapter 2.2.4. *	9-11	
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The present search report has been drawn up for all claims			
Place of search <b>THE HAGUE</b>		Date of completion of the search <b>25 March 1998</b>	Examiner <b>Kania, T</b>
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background G : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons \$ : member of the same patent family, corresponding document</p>			